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COFILIN NAVIGATES CELLULAR CYTOSKELETON AND INVASION RESPONSES TO TGF- β TOWARDS PROSTATE CANCER METASTASIS

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COFILIN NAVIGATES CELLULAR CYTOSKELETON
AND INVASION RESPONSES TO
TGF- β TOWARDS PROSTATE CANCER METASTASIS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine at the University of Kentucky

By

Joanne Collazo Santiago

Lexington, Kentucky

Director: Dr. Natasha Kyprianou, Professor of

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2013

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ABSTRACT OF DISSERTATION

COFILIN NAVIGATES CELLULAR CYTOSKELETON AND INVASION RESPONSES TO TGF- β TOWARDS PROSTATE CANCER METASTASIS

Cofilin's activity to nucleate actin filament assembly, is regulated by phosphorylation at a single site on the amino terminus, Serine 3. Phosphorylation at this site abolishes the ability of ADF/cofilin to bind to F-actin and inhibits its severing function. This work characterizes the ability of dephosphorylated cofilin (mutation at Serine 3 site) to navigate prostate cancer actin cytoskeleton and metastatic properties in response to TGF- β . TGF- β increased Lim Domain Kinase 2 (LIMK-2) activity leading to cofilin phosphorylation and decrease actin filament severing in wild type cofilin (WTCFL) PC-3 cells. Constitutively active cofilin in Serine 3 cofilin mutants (S3ACFL) promoted prostate cancer cell filopodia formation, actin severing and directed TGF- β mediated migration and invasion. Co-culture of prostate cancer cells with prostate cancer associated fibroblasts induced cell invasion in WTCFL and S3ACFL cells. Active cofilin further enhanced the invasive response, even in the presence of a TGF- β -neutralizing antibody, implicating the contribution of the microenvironment. Active cofilin led to a significant increase in prostate cancer cell metastatic potential *in vivo* and cofilin correlated with metastasis in a mouse model of prostate tumor progression. In human prostate cancer, cofilin expression was significantly higher in metastasis compared to the primary tumors. Cofilin thus emerges as a regulator of the actin cytoskeleton remodeling capable of coordinating the cellular responses to TGF- β towards prostate cancer metastasis. Understanding how cancer cells interpret TGF- β signals from the microenvironment, is critical for defining the mechanism via which TGF- β function is switched from a growth suppressor to a metastasis promoter. Here we show that in prostate cancer, TGF- β action is directed by active cofilin enabling actin cytoskeleton changes and metastatic behavior. The significant association of cofilin with prostate cancer metastatic progression supports its predictive and targeting value in metastasis.

KEY WORDS: Prostate Cancer, Cofilin, Actin Cytoskeleton, Filopodia, Metastasis

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.... to life “A la Vida”. And to all of us who live in love with life. Because science would not be possible without a real passion for life and all its wonders.

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CHAPTER I

INTRODUCTION

Normal Prostate Gland Anatomy and Function

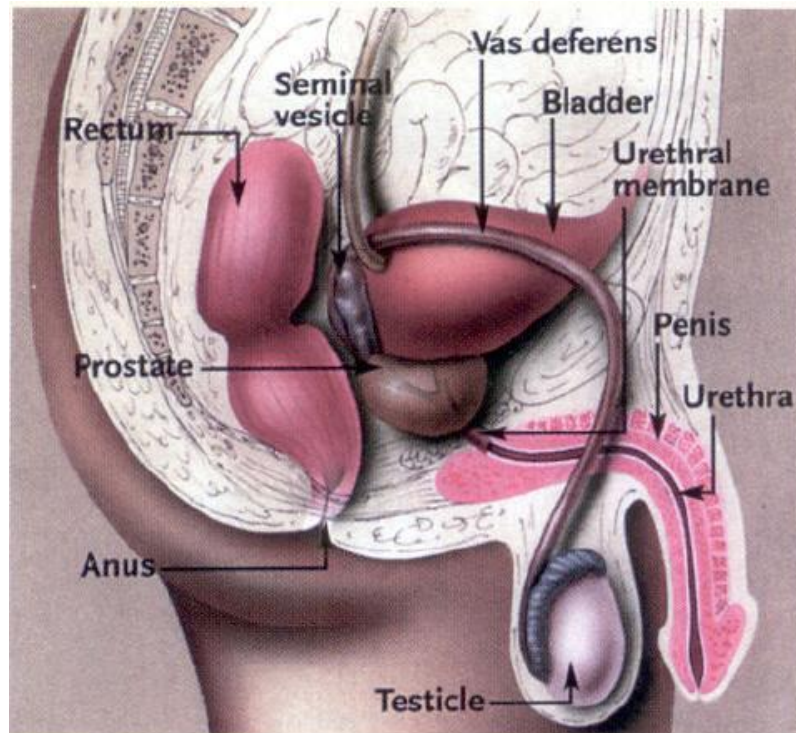
The prostate gland is an endocrine organ, located in the male pelvis, above the anus, below the urethra and it is composed of glandular and fibromuscular tissue (Figure 1.1) (Seisen T. et al.; 2012). The glandular cells are known to secrete the prostate specific antigen PSA which have been used for many years for the screening and early detection of prostate cancer. The prostate gland is essential for the process of fertilization, it is responsible for the secretion of protein rich seminal fluids and proteases like the prostate specific antigen (PSA) that nourishes and protect the sperm (Leissner & Tisell, et al.; 1979). These fluids are delivered into the urethra via prostate gland contractions where they are expelled with sperm as semen during the process of ejaculation.

The prostate gland, develops from the endodermal urogenital sinus (UGS), which is derived from the caudal terminus of the hindgut. Embryonic connective tissue known as urogenital sinus mesenchyme (UGM) surrounds the embryonic connective tissue. (Cooke 1991). The growth and development of the prostate gland is dependent on androgens. Androgens are mainly produced by the testes although a small amount of androgens are known to be produced by the adrenal gland (Yadav N. et al. 2012). Testosterone is the most predominant circulating androgen; in the prostate testosterone is converted to dihydrotestosterone (DHT) by the enzyme 5 α -reductase. Before sexual differentiation of the UGS, UGM expresses androgen receptors (AR) in both sexes and thus acquires the

capacity to undergo masculine development (Cooke et al. 1991). In response to fetal testicular androgens, epithelial buds are going to emerge from the wall of the UGS, grow into the surrounding UGM, and form the lobar subdivisions of the prostate into dorsal-lateral, ventral, and anterior prostate (Price, 1963, Sugimura et al. 1986 , Kinbara and Cunha 1995, Marker et al. 2003).

The androgenic effects on prostate development are mediated via androgen receptors (AR). The androgen receptor not only plays a critical role in the normal development of the prostate but in the process of prostate cancer development (Hodgson et al., 2012). This transcription factor regulates the expression of genes that are responsible for the modulation of epithelial cell growth, proliferation, survival, and differentiation (Cunha et al., 2004, Heinlein & Chang, et al. 2002). Upon steroid binding the AR suffers a conformational change which result in the dissociation of cytoplasmic chaperones revealing the nuclear localization signal. The steroid bound receptor then translocates to the nucleus, where it binds to DNA and interacts with transcriptional coregulators regulating the expression of targeted genes (Koochekpour, 2010). The AR is known to be expressed at some level in the majority of primary prostate cancers (Peter E. Lonergan, Donal J Tindal 2011). Studies have established a relationship between the AR expression levels in primary and metastatic progression to advanced castration resistant prostate cancer (Hodgson et al., 2012).

Figure 1.1 The Anatomy of The Prostate. The prostate, gland is located between the male bladder and the penis, in front of the rectum. The urethra runs through the center of the prostate, from the bladder to the penis, allowing urine flow out of the body. The prostate secretes fluid that nourishes and protects sperm. During ejaculation, the prostate transports this fluid into the urethra, where is expelled with sperm as semen.



From: © Trinidad and Tobago Prostate Brachytherapy Limited

Figure 1.1 The Anatomy of the Prostate

Prostate Cancer Development and Progression

Prostate cancer or adenocarcinoma of the prostate is defined as a tumor growing in the prostate gland (Figure 1.2). It is the most common form of cancer diagnosed in North America (According to the American Cancer Society). The American Cancer Society has estimated a number of 238,590 new cases of prostate cancer for 2013. It is also the second leading cause of male cancer related deaths in the United States and unfortunately 29,720 of these patients will die from the disease as a result of metastatic spread to the bone.

The tissue heterogeneity that characterizes prostate tumors has challenged the development of reliable biomarkers for prediction and prognosis of the disease. Today, there is a real need for the development of novel prostate cancer biomarkers that will translate in better methods for screening, therapeutic approaches and implementing prevention strategies. Identification of patients at ‘high risk’ for the development of metastatic prostate cancer will allow for the selection of those patients that will benefit from aggressive interventions to prevent progression and delay bone metastasis (Logothetis et al, 2013).

The main risk factor for prostate cancer is age. About 5-10% of prostate cancers can be attributed to gene defects, meaning that males with family history are more at risk of developing prostate cancer themselves. Although several genes have been identified to be involved in prostate cancer, the BRCA1 and BRCA2 genes are associated with having a two to five times higher risk (Agalliu, 2009, Castro and Eeles, 2012, Dumitrescu, 2012). A third risk factor for the development of prostate cancer is race; being African American

males at higher risk of developing prostate cancer than White or Hispanic males (Crawford 2003, Browley, 2012).

Prostate cancer progresses from prostatic intraepithelial neoplasia into metastatic and androgen independent carcinoma (Figure 1.2). The bone is the most common metastatic site of prostate cancer with 90% of patients with advanced prostate cancer developing skeletal metastasis. The normal prostate gland is encapsulated by two layers of epithelial cells known as the basal and luminal cell layers. As prostate cancer progresses, some cancer cells with genetic predisposition and promotion by environmental stimuli, become more aggressive, begin to degrade the surrounding double layer of epithelial cells and escape into the stroma, triggering a series of molecular events known as the metastatic cascade. The next step for this specific set of malignant cells is to directly invade the surrounding tissue, or disseminate via lymphatic system allowing the spreading of cancer cells, to those organs situated near the prostate such as the neurovascular bundle, penis, seminal vesicles, bladder and rectum. The intravasation of cancer cells into the lymphatic system result in the systemic spread of cancer cells to distant organs, including the bones, lung, and liver.

Oncogenes and Tumor Suppressors in Prostate Cancer

Among the molecular pathways known to be altered in cancer oncogene activation and tumor-suppressor suppression are the most common events triggering the initiation and progression of most types of cancers, however a relatively small number have been found to be implicated in prostate cancer. Some of the genes found to be altered in prostate cancer are the oncogene Myc and the product of *TMPRSS2:ETS* gene fusions which are

often elevated throughout the course of disease progression. In addition, the PI3K/AKT/mTOR pathway is commonly activated in prostate cancer due to the loss of tumor-suppressor *PTEN*, while *RB* activity and/or expression loss have been found to promote castration resistant prostate cancer.

PI3K activity plays a critical role in the regulation of cell growth and apoptosis. It is known that about 40% of primary prostate cancers and 70% of metastatic prostate cancers present gene alterations involving the PI3K/AKT pathway such as the loss of the *PTEN* (phosphatase and tensin homolog), encoding a lipid phosphatase that negatively regulates AKT activation (El Sheikh et al., 2008). Studies involving homozygous deletion of PTEN in mouse models show that the loss of PTEN is sufficient to induce metastatic tumors (Wan et al., 2003). However recent data have shown that PTEN deletions in mouse knockout models are not sufficient to support progression into metastatic prostate cancer, and suggested the involvement of additional molecular mechanisms supporting metastatic spread including the AKT PI3K and TGF- β signaling (Bjerke et al. 2013).

Figure 1.2 Prostate Cancer Progression. Prostate cancer progresses from prostatic epithelial neoplasia into metastatic and androgen dependent carcinoma. As prostate cancer progresses, some cancer cells with genetic predisposition and promotion by environmental stimuli, become more aggressive, begin to degrade the surrounding double layer of epithelial cells and escape into the stroma.

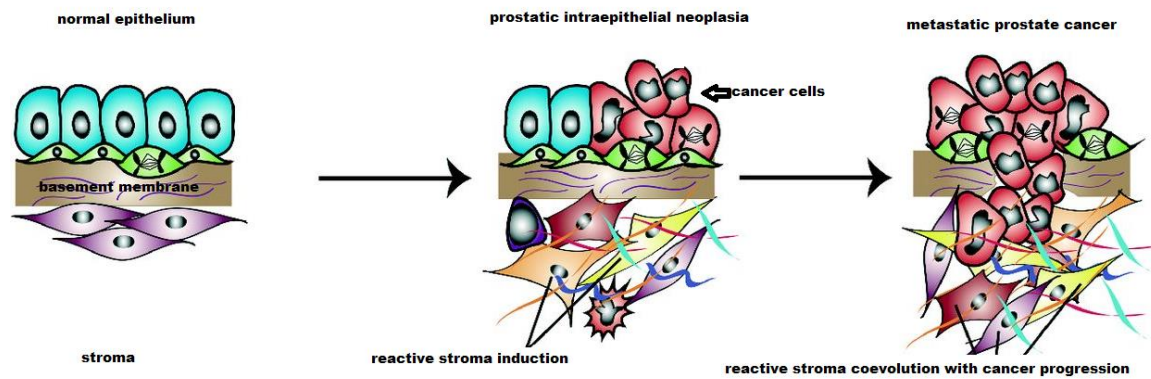


Figure modified from: Barron D A, and Rowley D R Endocr Relat Cancer 2012; 19:R187-R204

Figure 1.2 Prostate Cancer Progression

Current Therapeutic Strategies For Prostate Cancer

Among the available prostate cancer therapies endorsed by the clinical practice; surveillance, radical prostatectomy and external-beam radiotherapy are offered to men with clinically localized prostate cancer (Thompson et al., 2007, Heidenreich, 2011). Radical prostatectomy and radiation therapy are the most commonly used treatments for organ confined tumors with a 10 year survival rate of 60%. However several side effects including sexual, urinary and bowel dysfunction can affect the patient quality of life. The only treatment conferring improved survival to patients with advanced metastatic prostate cancer is androgen ablation therapy. The apoptotic response to androgen ablation is the underlying mechanism driving tumor regression and therapeutic benefit in prostate cancer patients (McKenzie and Kyprianou, 2006). The majority of prostate tumors recur however emerging as castration-resistant, due to prostate cancer cells developing resistance to androgen-ablation induced apoptosis.

Evolution from androgen dependent to androgen- independent prostate cancer (currently known as castration-resistant prostate cancer) is the result of a complex interplay of signaling pathways becoming dysfunctional, triggered by an aberrant AR signaling (Lonergan and Tindal 2011). Several mechanisms have been proposed to lead AR mediated castration resistance such as: 1) AR amplification/overexpression; 2) gain of function; 3) intracrine androgen production 4) overexpression of AR cofactors; 5) ligand independent AR activation by cytokines and growth factors; 6) constitutive active messenger ribonucleic acids (mRNA) splice variants of AR. Molecular studies involving immunohistochemical profiling of AR on tumor tissue samples from castration resistant patients have shown that AR is not only highly expressed, but also transcriptionally

active (Ruizeveld de Winter et al., 1994, Taplin et al., 1995, Holzbeierlein et al., 2004). In a similar way, studies in xenograft models also showed an increased in the expression of AR and restoration of its activity in those tumors that relapsed after castration (Gregory et al., 1998, Chen et al., 2004). These findings, together with studies involving RNA interference confirm that AR was still required for growth in castration resistant prostate cancer (CRPC). The high levels of androgens in samples from castration resistant prostate cancer patients (Mohler et al., 2004, Titus et al., 2005), and compelling evidence showing that tumors had increased expression of androgen synthesis enzymes, (Stanbrough et al., 2006, Montgomery et al., 2008), implicate a castration resistance mechanism, via which tumor cells are responsible for androgen synthesis, leading to the reactivation of AR transcriptional activity and prostate cancer growth and progression.

Metastasis and The Tumor Microenvironment

The process of cancer metastasis is facilitated by interactions between cancer cells and the tumor microenvironment (Fang and Declerck, 2013 Jezierska-Drutel, 2013, Astekar et al., 2013). These interactions are mediated by growth factors secreted not only by cancer cells but from additional components of the tumor microenvironment such as immune cells, endothelial cells and fibroblasts (Figure 1.3) (Dutsch-Wicherek and Kazmierczak, 2013). The contribution of growth factor secretions by different cell types present in the tumor microenvironment enables the optimal conditions for the growth and proliferation of cancer cells. For example, it is known that in breast cancer, parathyroid hormone-related protein and interleukin (IL)-11, produced by cancer cells, activate osteoclasts by the receptor activator of nuclear factor- κ B (RANK)-RANK ligand

(RANKL) (Morgan et al., 2004). The activated osteoclasts are responsible for the induction of bone-derived factors release. The molecular mechanisms responsible for prostate cancer bone metastasis are not fully understood. However recent studies have shown that the co - culture of LNCaP cells with mouse stromal MC3T3-E1 cells result in the induction of osteoblastic differentiation of MC3T3-E1 cells. Furthermore, it have been shown that LNCaP cells and BMP-4 can cooperatively induce the production of growth factors, such as fibroblast growth factor (FGF)-2 and epidermal growth factor (EGF) in MC3T3-E1 cells, promoting proliferation of LNCaP cells (Nishimori et al., 2012). These stromal cancer cell interactions were found to not only favor the survival of prostate cancer cells, but also induce the differentiation of bone stromal cells in the bone microenvironment, facilitating the osteoblastic metastasis of prostate cancer (Nishimori et al., 2012). These compelling findings implicate a direct contribution of the bone microenvironment in facilitating the colonization of prostate cancer cells into the bone. In an attempt to explain this organ specific pattern of metastatic spread not only observed in prostate cancer but among different types of cancers, the “seed and soil” theory was proposed over a century ago (1889) by Stephen Paget. The theory proposed that there was something rich in the metastatic sites that promoted cancer cell growth in a similar way that seeds tend to grow in fertile soil, i.e., that factors in the environment at a metastatic site contributed to the targeted growth and proliferation of cancer cells. (Stephen Paget, 1889, reviewed in Tantivejkul et al. 2004). Today this theory continues to be relevant and supported as many factors have been found to be involved in the attraction and growth of prostate cancer cells specifically to the bone. As an example reflecting the important role of the bone microenvironment in supporting the growth and

proliferation of prostate cancer cells, bone extracts are able to induce at least a three-fold increase in invasion by PC-3 and DU145 cells compared with brain and other tissue extracts, which demonstrates that bone contains significant migration and chemoinvasion promoting factors for prostate cancer cells (Jacob et al., 1999). It is known that more than 95% of the bone ECM is composed of collagen type I, however, many other proteins are also deposited by osteoblasts during bone formation (Hauschka et al., 1986). Osteonectin, a glycoprotein known to be secreted by osteoblasts during bone formation, has been identified as the chemoattractant responsible for the promotion of prostate cancer cell invasion (Jacob et al., 1999). In addition co-culture of PC-3 cells with osteoblasts have revealed that TGF- β produced by osteoblasts stimulates PC-3 cell migration and invasion as well as increases $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins expression (Festuccia et al., 1999).

The signaling transduction and cell to cell interactions contributing to prostate cancer cell proliferation in the bone, are mediated through the engagement of integrin receptors since the main component of bone ECM is collagen type I which is a ligand for $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins. Indeed greater proliferation rates for prostate cancer cells have been observed in cells grown on collagen I compared to fibronectin substrates; cell signaling through phosphatidylinositol 3-kinase (PI3K) and increased expression of cyclin D1 are implicated in this process (Kiefer and Farach-Carson, 2001). Blocking antibodies against the $\alpha v\beta 3$ integrins can effectively reduce prostate cancer cell adhesion to crude bone protein extract by 94% (Hullinger et al., 1998), suggesting an important role of the integrins in the process of bone metastasis.

Aside from the contribution of factors in the bone microenvironment, prostate cancer cells themselves are known to be functionally involved in bone remodeling (Chung, 2003). Prostate cancer metastases are known to have an osteoblastic nature, and evidence supports a role of bone morpho- genetic proteins (BMPs) in the course of bone metastasis since they contribute to bone formation. In 1992, Bentley et al. reported for the first time the expression of BMP-6, a member of the TGF- β superfamily, in prostate tissue samples of over 50% of patients with clinically defined metastatic prostate cancer, but not non-metastatic or benign prostate samples. Overexpression of BMP-6 in metastatic prostate cancer cells was confirmed in subsequent studies by, (Barnes et al., 1995; Hamdy et al., 1997; Autzen et al., 1998; Thomas and Hamdy, 2000). The secretion of BMP-6, among other proteins, by prostate cancer cells could explain targeted osteoblastic lesions during prostate cancer metastasis since BMP-6 stimulates osteoblastic differentiation of pluripotent mesenchymal (Ebisawa et al., 1999). Moreover, osteoblastic differentiation is dependent on the activation of focal adhesion kinase (FAK), an immediate effector of the integrin signaling pathway (Tamura et al., 2001).

Figure1.3 Components of The Tumor Microenvironment. The process of cancer metastasis is facilitated by interactions between cancer cells and the tumor microenvironment. These interactions are mediated by growth factors secreted by cancer and additional components of the tumor microenvironment. The recruitment of endothelial cells, immune cells and CAFs facilitates ECM degradation and the escape of cancer cells into the stroma.

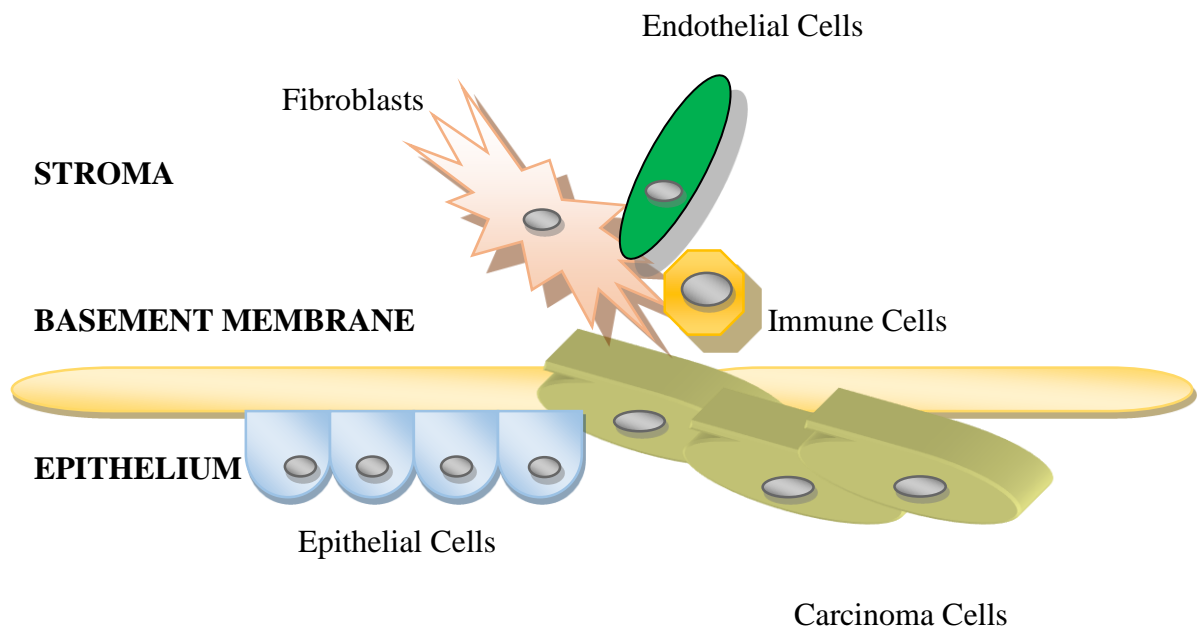


Figure 1.3 Components of The Tumor Microenvironment

TGF- β Signaling in The Prostate Microenvironment

Transforming growth factor- β (TGF- β) and its signaling effectors are extensively studied as critical determinants of tumor cell behavior in the context of the microenvironment. This secreted cytokine was originally named from its capacity to induce anchorage-independent growth of normal rat kidney cells and fibroblast cell lines i.e., to induce transformation (Moses et al. 1981, Roberts et al. 1981). The later discovery of its multifunctional roles, not restricted to cell growth regulation, but also involving, cell cycle control, differentiation, apoptosis, extracellular matrix formation, angiogenesis, and immune functions, proved the name of Transforming Growth Factor Beta to be misleading (Bottner et al. 2000, Dunker and Krieglstein et al. 2000, Lawrence et al. 1996).

Three different types of mammalian TGF β 's have been characterized so far, TGF- β 1, TGF- β 2 and TGF- β 3. The most frequently upregulated in tumor cells is TGF- β 1 (Schirmer et al. 2009). TGF β 's signaling is transmitted through two types of receptors (T β RI and T β RII) followed by downstream targeting through regulation of the SMAD family of protein effectors. T β RI and T β RII are serine-threonine proteins kinases that contain an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic serine-threonine kinase domain (Figure 1.4). Type one receptors have a GS domain preceding the kinases domain which is phosphorylated during signal transduction. Type II receptors have a shorter C-terminal tail at the end of the kinase

domain and have also a shorter extracellular domain with different distribution of conserved cysteines. During TGF- β signal transduction, type II receptor goes through a conformational change upon ligand binding of the extracellular domain and forms a complex with the type I receptor which facilitates phosphorylation of the type I receptor at its GS domain. Once phosphorylated by T β RII, T β RI phosphorylates the receptor activated SMAD proteins (SMAD2 and SMAD 3), which form a heteromeric complex with the co- SMAD SMAD 4 and enter the nucleus to activate or suppress target gene expression by direct or indirect interactions with promoter regions of specific genes (Fig. 1.4). TGF- β signal transduction results in the inhibition of cell entry into S - phase by upregulation of cyclin-dependent kinase (CDK) inhibitors (Padua and Massague, 2009). A critical event involved in TGF- β mediated growth arrest is induction of expression of the CDK inhibitors p15 and p21. Induction of p15 or p21 in response to TGF- β is due to SMAD- mediated transcriptional activation. p15 prevents cell cycle progression by interacting with complexes of CDK2 and cyclin A or cyclin E and inhibiting CDK2 kinase activity. The second inhibitor, inhibitor p21 inactivates the catalytic activity of CDKs by interacting and inactivating CDK4 and CDK6, or by associating with cyclin D complexes of CDK4 and CDK6. Without the action of CDKs and formation of active-cell cycle promoters cells cannot progress through the cell cycle. TGF- β has been shown to induce apoptosis in many cell types (Gordon and Blobel, 2008).

The SMAD pathway is the classical signaling pathway of TGF- β family members signaling. Increased levels of SMAD 3 or SMAD 4 induce apoptosis, and dominant negative/loss of SMAD 3 protects against cell death (Fig. 1.4). Taken together these findings implicate SMAD signaling as the main pathway of TGF- β mediated apoptosis.

The proapoptotic genes involved in SMAD signaling have not yet been identified. Another pathway involved in this process is down regulation of Bcl-X and activation of caspase 3 and 8 (Weinberg 1989). Deregulation TGF- β signaling contributes to tumorigenesis by either loss of expression or mutational inactivation of its membrane receptors or intracellular effectors, the SMADS. Inactivation mutations in T β RII, SMAD 2 and SMAD 4, are commonly found in many types of human cancers including, brain, lung, breast, ovarian and pancreatic cancer. In most of colon and gastric tumors with microsatellite instability, T β RII is inactivated by a functional mutation and in a smaller percent for microsatellite stable colon cancers (Myeroff et al. 1995). In many other types of human cancers like pancreatic, liver and endometrial cancer with microsatellite instability T β R-II mutations are relatively rare (Gille et al. 2001). In the case of breast cancer no mutations or structural dysfunctions of T β R-II have been found, but a limited receptor expression correlates with loss of TGF- β responsiveness, while TGF- β growth inhibition is restored in breast cancer cells by stably expressing T β R-II. TGF- β inhibits the outgrowth of carcinomas in situ during the early stages of breast cancer while it promotes tumor progression and enhances the ability of cancer cells to metastasize during late stages of the disease. (Ghellal et al. 2000).

In addition to the SMAD mediated signaling there are other known mechanisms by which TGF- β can perform multiple functions like for example the ERK, JNK, MAPK, P13 and Rho GTPase pathways. Evidence from our laboratory and by others, have established that TGF- β signaling can proceed without the principal effectors of the signaling pathway SMAD 2 and 4 (Zhu, 2006). Studies involving induced apoptosis in HT58 lymphoma cells do not reveal any impact in apoptosis (Teicher, 2007). Moreover a proteomic based

approach identified two proteins, prohibitin and cofilin as intracellular effectors of TGF- β in human prostate cancer cells (Zhu, 2006).

TGF- β A Major Contributor To Prostate Tumorigenesis

Elevated TGF- β expression in the prostate tumor microenvironment is functionally linked to tumor progression by increasing angiogenesis and decreasing immune responses (Teicher. et al. 2007). TGF- β has shown pro-oncogenic functions in host immune cells, myeloid immune suppressor cells (MISCs) by enhancing their ability to infiltrate tumors and promote new tumor vessel formation. Studies using transgenic mice engineered with a deleted gene encoding for a TGF- β receptor indicated that TGF- β switch from a tumor suppressor to a tumor promoter phenotype could involve the recruitment of MISCs into the tumor microenvironment, probably due to increased production of chemokine CXCL5 or SDF-1/CXCR4 cytokine system by the tumor cells.

In normal prostate cancer cells and prostatic carcinoma cells TGF- β is known to induce apoptosis *in vitro* and *in vivo*. Our laboratory have recently dissected the contribution of TGF- β signaling in the *in vivo* prostate tumor microenvironment, using the DNT β RII/TRAMP transgenic mouse model as a tool for the characterization of the *in vivo* consequences of an inactivated TGF- β 1 signaling on prostate tumor initiation and progression. This work established that a dysfunctional TGF- β 1 signaling mechanism results in partial loss of the inhibitory effects of TGF- β 1 leading to an increase of prostate cancer epithelial cell proliferation and angiogenesis thus promoting tumor progression (Pu et al., 2009).

The role of TGF- β in the inhibition of epithelial cell proliferation and promotion of apoptosis in normal prostate epithelium and in early prostate cancer (Bello- De Ocampo and Tindall et al., 2003). The fact that disruptions of TGF- β signaling due to either loss or inactivation of its transmembrane receptors or intracellular effectors can lead to prostate tumor growth rendered this cytokine to be a potential therapeutic target.

The challenge in studying TGF- β as a potential therapeutic agent relies in the ability of this cytokine to not only suppress cancer cells growth and proliferation during the early stages of cancer, but to promote tumor progression and metastases during the advance stages of the disease (Padua et al., 2009). TGF- β increased expression in the tumor microenvironment provides an advantage for tumor cell survival, because of the angiogenic capacity of TGF- β and its potent immunosuppressive effects, including the inhibition of natural killer cells and lymphocyte-activated killer cells. Experimental studies with pancreatic cell lines (Panc-1) revealed a significant correlation between the level of expression of TGF- β 1 and lymph node involvement as well as depth of invasion. Increased TGF- β levels support its involvement in the process of epithelial to mesenchymal transition (EMT) and enhancement of the ability of invasion of pancreatic cancer cells (Yin et al., 2006).

Transgenic adenocarcinoma of the prostate mouse model (TRAMP), a prostate cancer transgenic mice harboring the dominant-negative mutant TGF- β type II receptor (DNTGF β RII), manifested early malignant changes and subsequently highly aggressive prostate tumors at a younger age, compared to control TRAMP+/Wt TGF β RII mice (Pu et al. 2009). These prostate tumors exhibited significantly increased proliferation and vascularity. An epithelial-mesenchymal transition (EMT)-effect was also detected in

prostates of TRAMP+/DNTGF β RII mice, as documented by the loss of epithelial markers (E-cadherin and β -catenin) and upregulation of mesenchymal markers (N-cadherin) (Pu et al., 2009). Thus *in vivo* disruption of TGF- β signaling accelerates the pathological changes in the prostate by altering the kinetics of prostate growth and inducing EMT.

During early stages of tumorigenesis for prostate, breast and colon cancer, TGF- β acts as a potent tumor suppressor and has an antiproliferative effect, inhibiting tumor growth, but as the disease progresses to advance metastatic stage, there is a TGF- β switch to tumor promoter, exerting a proliferative effect, increasing motility and cancer spread. The dual role of TGF- β presents a serious clinical challenge in the treatment of many types of cancers since TGF- β can dictate the dynamics of the therapeutic response and metastatic spread in human cancer. Even though the therapeutic targeting of TGF- β signaling have proven to be very challenging; today neutralizing antibodies and small molecule inhibitors against TGF- β have been developed and implemented in clinical trials. However the success of these promising therapeutical agents still relies in the better understanding of the functional switch of TGF- β from a tumor suppressor to a lethal tumor promoter.

Angiogenesis, the formation of new blood capillary networks sprouting from pre-existing vessels is an important and active process during embryonic development. During cancer progression the development of new vasculature nourishes and facilitates the growth of primary tumors favoring their progression and metastasis. Under normal circumstances of angiogenesis is active during embryonic development and growth but becomes quiescent at adult stages; however reactivation can occur during wound healing

and under different pathological conditions including tumor growth and metastasis. TGF- β is known to suppress or stimulate angiogenesis in a context dependent manner. The main mechanisms by which TGF- β enhances or suppresses angiogenesis is via regulation of vascular growth factor (VEGF). In vascular endothelial cells TGF- β can induce expression of vascular VEGF via the promotion of fibroblast growth factor FGF-2 which upregulate VEGF synthesis (Markowitz et al., 1995). Upon the induction of expression of VEGF by TGF- β , VEGF can activate VEGR1 and VEGR2 which are two types of tyrosine kinase receptors (Byrne et al., 2005). VEGR2 is involved in endothelial cell proliferation and survival while VEGR1 have been implicated in chemotaxis and vascular permeability (Gille et al., 2001). Several studies documented a correlation between TGF- β 1 overexpression with enhanced angiogenesis around the tumor mass, leading to an increased frequency of metastasis of prostate cancer cells (Roberts et al., 1991). In contrast, TGF- β secreted by gallbladder tumors inhibits angiogenesis and reduces tumor growth, suggesting its tumor suppressor function (Weinberg, 1989). The biggest challenge in understanding this TGF- β “paradox” resides in elucidating those tumorigenic signaling pathways by which normal tissue specification become aberrant and which of the reciprocal signaling events mediated by TGF- β cooperate to control the microenvironment. Interestingly the majority of TGF- β mutations that lead to a dysfunctional TGF- β signaling do not affect primary tumors but can enable cancer cells to acquire EMT and invasive characteristics.

During the process of oncogenic EMT, clusters of malignant cells, loose their epithelial characteristics and acquire self-sustained migratory and highly invasive cell phenotypes. Thus EMT is characterized by loss of proteins associated with polarized epithelial

phenotype and *de novo* synthesis of proteins associated with mesenchymal, migratory morphology of transitioning cells. As an example, the loss of epithelial proteins such as MUC1, E-cadherin, ZO-1, desmoplakins, and cytokeratin 18, in cells of epithelial units defines epithelial dedifferentiation. In contrast *de novo* expression of vimentin is correlated with down modulation of epithelial cytokeratins and has been proposed as canonical marker of the fibroblastoid state of transitioning cells (Franke et al., 1982; Boyer et al., 1989). The specific molecular mechanisms by which TGF- β induces EMT *in vivo* has not been well established, but studies *in vitro* have indicated to be dependent of both, Smad- and no- Smad signaling pathways. Our group demonstrated an epithelial-mesenchymal transition (EMT)-effect in prostates of TRAMP+/DNTGF β RII mice (bearing a mutant nonfunctional TGF- β receptor II), as documented by the loss of epithelial markers (E-cadherin and β -catenin) and upregulation of mesenchymal markers (N-cadherin) [19]. Thus *in vivo* disruption of TGF- β signaling accelerates the pathological changes in the prostate by altering the kinetics of prostate growth and inducing EMT. However, overexpression of the type I TGF- β receptor mutant that lacks the ability to bind SMAD2/3 but is capable of activating non-Smad signaling, was associated with increased tumorigenicity of the primary tumors, but decreased metastatic potential of xenografted breast cancer cell lines (Tian et al., 2003; Tian et al., 2004) . TGF- β signals in the same fashion, suggesting that TGF- β signaling may not necessary be permanently or constitutively active at each stage of early tumor development, but rather it depends on the specific stage of the disease, the tumor cell type and the surrounding microenvironment.

Figure 1.4. SMAD dependent TGF- β Signaling Pathway. During TGF- β signal transduction, type II receptor goes through a conformational change upon ligand binding of the extracellular domain and forms a complex with the type I receptor which facilitates phosphorylation of the type I receptor at its GS domain. Once phosphorylated by T β RII, T β RI phosphorylates the receptor activated SMAD proteins (SMAD2 and SMAD 3), which form a heteromeric complex with the co- SMAD SMAD 4 and enter the nucleus to activate or suppress target gene expression by direct or indirect interactions with promoter regions of specific genes.

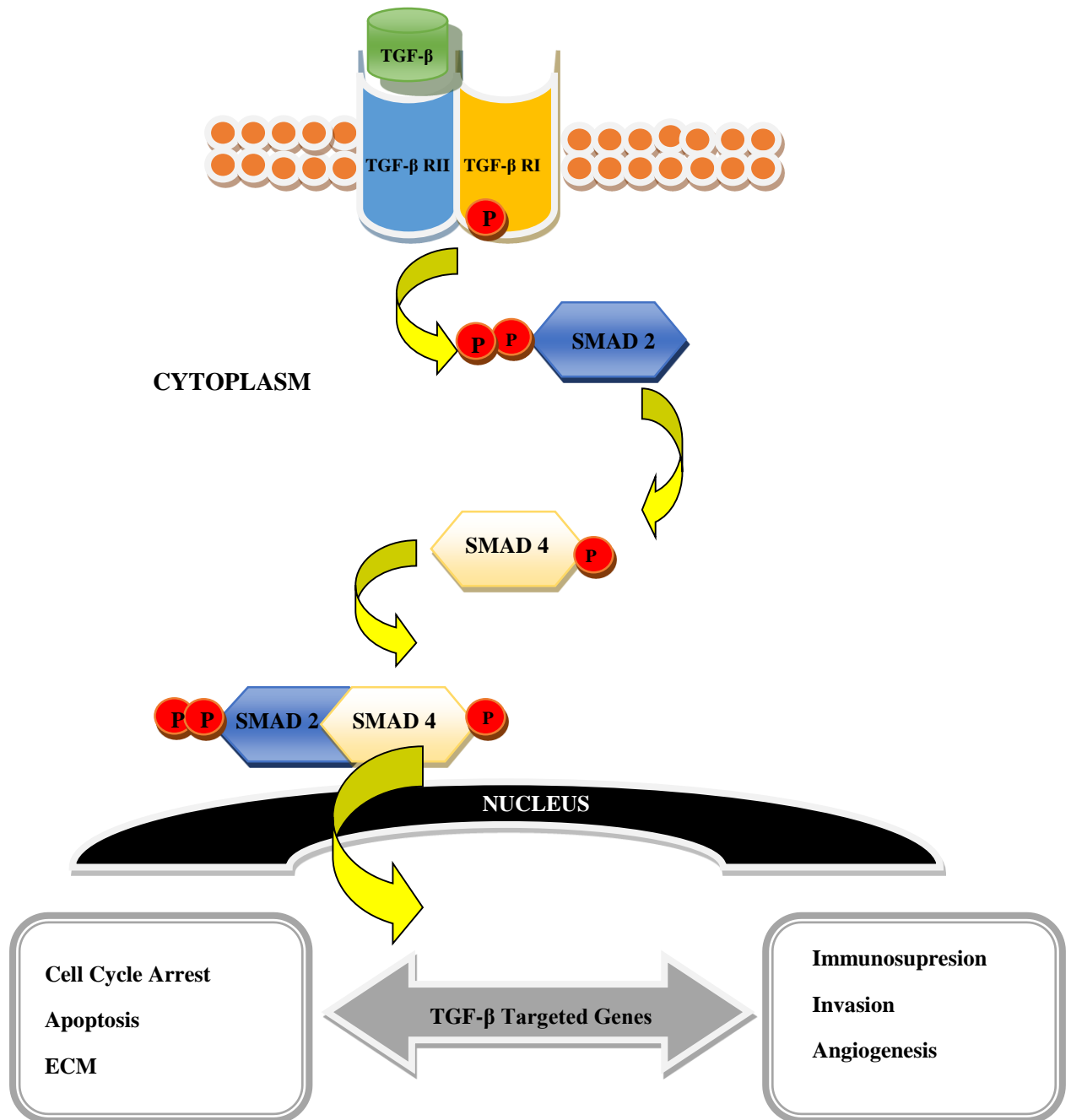


Figure 1.4 SMAD dependent TGF- β Signaling Pathway

The Role of TGF- β in Prostate Cancer Metastasis

The first and most critical step of tumor metastasis is the detachment of cancer cells from the primary tumor and extracellular matrix (ECM) following invasion into the surrounding tissue, resistance to anoikis, and migration via a chemoattractive path to a metastatic site (Sakamoto and Kyprianou, 2010). A distinct molecular program is responsible for the regulation of the adhesion, migratory and invasive properties of disseminating tumor cells, all processes impacted by the dynamics of the cytoskeleton. Growth factors in the tumor microenvironment have been shown to modulate the activation of molecular pathways leading to the remodeling of the actin cytoskeleton. Growing evidence suggests that TGF- β reduces tumor cell adhesion, and cooperates with ErbB2 receptors to induce migration and invasion. The metastatic phenotype in cancer is primarily dictated by ECM degradation and formation, and dramatic changes in cell adhesion and mobility of cancer cells (Pardali and Moustakas, 2007). TGF- β can modulate such cell- interactions with ECM and several studies have demonstrated that TGF- β increases metastatic prostate cancer cell adhesion. TGF- β enhanced cell adhesion is mechanistically dependent upon p38 MAP Kinase activity. P38 MAP kinases are upregulated by TGF- β an action that modulates TGF- β dependent extracellular matrix degradation in osteoblasts (Kleef and Korc, 2002). The mechanism via which p38 MAP kinase regulates TGF- β signaling is believed to be at the level of SMAD activation via regulation of SMAD phosphorylation (Markowitz et al., 1995). Induction of p38 MAP kinase by TGF- β activates SMAD signaling effectors suggesting a possible mechanism for SMAD-dependent regulation of cell adhesion. Our group identified the actin binding

protein cofilin to be a SMAD-independent intracellular effector of TGF- β in prostate cancer cells (Zhu et al. 2006).

The Actin Cytoskeleton

The cellular cytoskeleton is composed of three main types of filaments, actin filaments, microtubules and intermediate filaments. While microtubules and actin filaments are directly targeted to giving structural support to the cell, intracellular trafficking and signaling; the actin filaments are directly responsible for cell movement. Actin is essential for cell survival because of its central role in key biological processes such as cell division, movement, structural and mechanical support. (Pollard and Cooper, 2009). This protein was first isolated from muscle cells and initially thought to be exclusively involved in muscle contraction. Accounting for about 5 to 10% of total protein, actin is known to be one of the most abundant proteins in all eukaryotic cells (Dominguez and Holmes, 2011). At least six different actin genes are known to encode actin in mammals, four of these genes are expressed in muscle and two in non-muscle cells. Three main actin isoforms expressed in vertebrates include, three α -isoforms of skeletal, cardiac, and smooth muscles and the β - and γ -isoforms expressed in nonmuscle and muscle cells. Actin proteins are very similar in amino acid sequence and highly conserved through evolution. Compared to eukaryotic cells, yeast have only one single actin gene, although 90% identical in amino acid sequence to mammalian actin. Some of the studies in model organisms including *Drosophila* (Wagner et al., 2002 and *C. elegans* MacQueen et al., 2005) have provided evidence to suggest that actin isoforms have both overlapping and

unique cellular functions. Table 1 is a collective summary of studies in mouse models with individual actin-isoform knockouts showing the diverse phenotypes indicating overlapping and non-overlapping functions between the different isoforms (Hoboken, 2010).

The individual actin protein is known as (G) or monomeric actin. Each of these globular actin monomers has binding sites that mediates head to tail interactions with two other actin monomers generating polymers of actin monomers called (F) or filamentous actin. Due to a rotation of 166° of each monomer in the actin filament resembles a double – stranded helix with all the actin monomers oriented in the same direction. Since G-actin is not an effective ATPase, whereas F-actin is, the main factor regulating the transition between G- and F-actin is nucleotide hydrolysis by F-actin, as the ATP state is more stable than the ADP state. Actin monomers join the fast-growing barbed (or +) end of the filament in the ATP state, hydrolysis takes place in the filament, and ADP-actin monomers dissociate faster from the pointed (or –) end. This mechanism of actin polymerization/depolymerization is known as actin filament treadmilling (Figure 1.5) (Wegner and Isenberg, 1983, Bravo-Cordero, 2013).

| Protein ablated | Gene | Allele | Transgenic rescue | Phenotype | References |
|-------------------------|-------|-----------------------------|------------------------|---|---|
| α skeletal-Actin | Acta1 | Null | | Pups die by 9 days of age; exhibit muscle weakness | Crawford et al., 2002 |
| | | | α cardiac-Actin | Full rescue | Nowak et al., 2009 |
| | | | γ cyto-Actin | Does not rescue | Jaeger et al., 2009 |
| α cardiac-Actin | Actc1 | Null | | Embryonic/perinatal death; disorganized myofibrils | Kumar et al., 1997 |
| α cardiac-Actin | | | γ smooth-Actin | Partial rescue of lethality; hearts defective | Kumar et al., 1997 |
| α smooth-Actin | Acta2 | Null | | Viable; defects in vascular contractility and blood pressure regulation | Schildmeyer et al., 2000 |
| β cyto-Actin | Actb | Hypomorph | | Embryonic lethal | Shawlot et al., 1998; Shmerling et al., 2005 |
| γ cyto-Actin | Actg1 | Null | | Reduced viability; small size; progressive deafness | Belyantseva et al., 2009; Bunnell and Ervasti, 2010 |
| γ cyto-Actin | | Conditional-skeletal muscle | | Progressive centronuclear myopathy | Sonnemann et al., 2006 |

Table 1. Actin Isoforms - Phenotypes in Mouse Models

Figure 1.5 Actin Treadmilling Protrusion Model. In a process known as treadmilling, actin subunits coming from filament depolymerization at the rear of the cell are recycled back into new filaments assembled at the front. It is the continuous assembly of actin filaments at the leading edge of cells what generates the protrusive extensions of the cytoplasm.

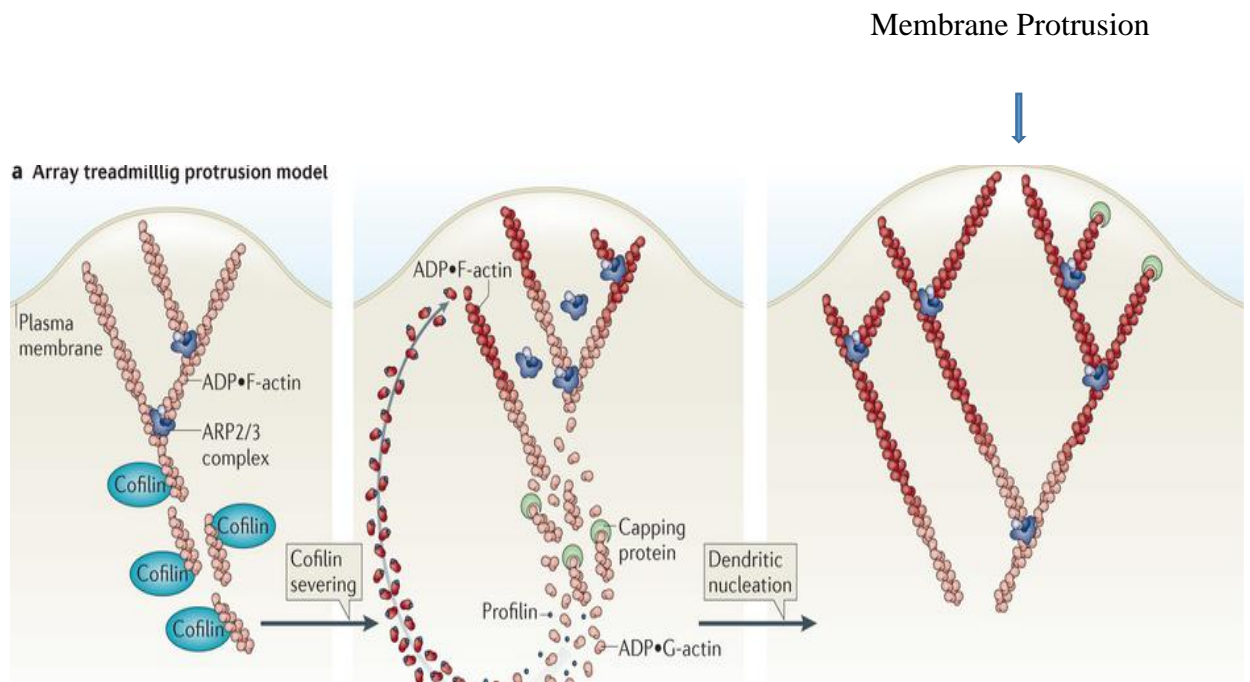


Figure Modified From: Nature Reviews Molecular Cell Biology (2013)

Figure 1.5 Actin Treadmilling Protrusion Model

Cytoskeletal Actin Remodeling and Cell Movement

Among the many critical functions of the cellular actin network cell movement is required as early as in embryonic development for the processes of morphogenesis, neurite movement in the developing nervous system, chemotactic movement of immune cells and wound healing (Gurniak et al. 2005). Without actin remodeling and cell movement the development and survival of multicellular organisms wouldn't be possible in any tissue environment. In a process known as treadmilling, actin subunits coming from filament depolymerization at the rear of the cell are recycled back into new filaments assembled at the front. It is the continuous assembly of actin filaments at the leading edge of cells what generates the protrusive extensions of the cytoplasm allowing the cell to move and determining the direction of cell movement. The dynamic net of actin filaments and its rapid polymerization and depolymerization at the leading and rear ends allow the cell to attach to a substrate, contract its body, push forward and move in response to stimuli from the microenvironment (Figure 1.6). (Yilmaz and Christophori, 2010).

The actin cytoskeleton remodeling network is composed by a series of actin binding proteins which cooperatively bind, nucleate, sever, and incorporate new actin monomers into new actin filaments allowing the cell to develop specific movement, adhesion, and invasion structures such as lamellopodia, filopodia and invadopodia (Figures; 1.6 and 1.7). Inside the cellular cytoskeleton actin is known to interact with more than 60 actin-binding proteins (ABPs) which regulates the assembly and disassembly leading to the remodeling of actin cytoskeletal networks. Cells extending their leading end depend

mainly on the activity of four core actin remodeling proteins: 1) The Arp2/3 complex, mediating the initiation of new filaments as branches on preexisting filaments (Pollard and Beltzner, 2002); 2) capping proteins which terminate filament growth (Cooper and Shafer, 2000); 3) ADF/cofilin severing actin filaments and promoting branching and depolymerization of existing actin filaments (Bamburg et al. 1999) and 4) profilin catalyzing the exchange of ADP to ATP, refilling the pool of ATP actin monomers and elongating the filaments (Shluter et al. 1997).

Figure 1.6 Cytoskeletal Remodeling and Cell Movement Structures. The dynamic net of actin filaments and its rapid polymerization and depolymerization at the leading and rear ends allow the cell to attach to a substrate, contract its body, push forward and move in response to stimuli from the microenvironment.

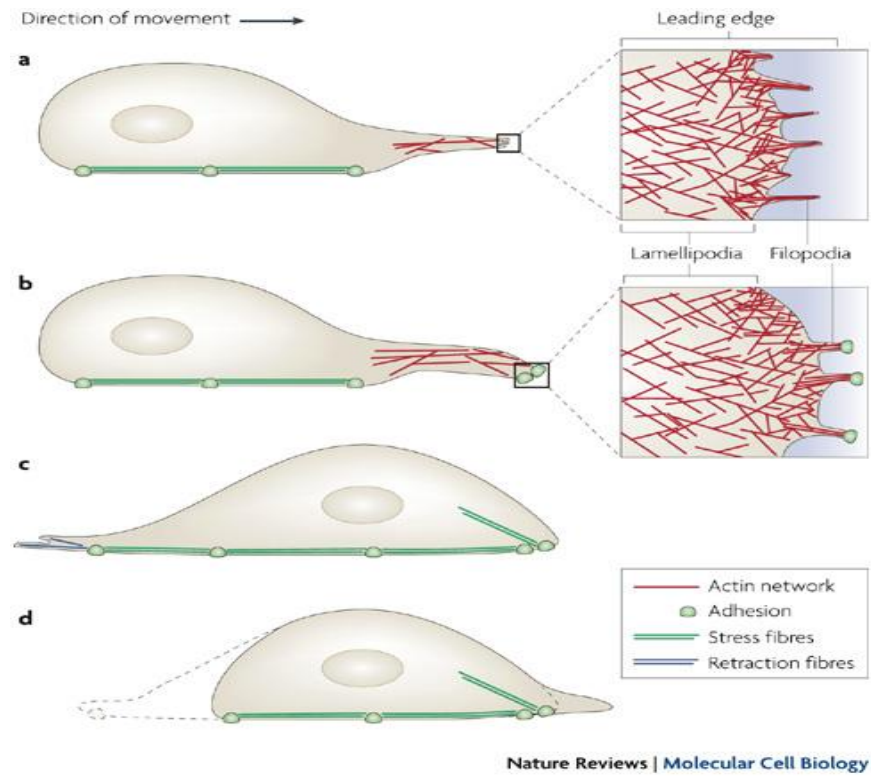


Figure 1.6 Cytoskeletal Remodeling and Cell Movement Structures

Cofilin in Control of Cell Movement

Among the key actin remodeling proteins needed for cytoskeletal reorganization at the leading edge of moving cells, cofilin have proven to be the main regulator of the actin dynamics since de novo synthesis of actin filaments in the cell is not favorable due to the instability of actin dimers and trimmers.

The binding of cofilin to the ADP- actin filament induces stress to the actin filament, changes the twist of the actin filament and promotes severing and disassembly of the filament (Bamburg, 1999). Cofilin severing activity on already existing filaments allowed free barbed ends for the rapid addition of new actin monomers resulting in the rapid growth of filaments and filament branching (Figure 1.7). It is known that the rapid polymerization of actin filament on a compartmentalized cell cannot continue without rapid depolymerization, thus in moving cells cofilin severing activity is critical for the formation of new actin filaments needed for the development of specific migration structures and Bamburg , 2010). Indeed ADF/Cofilin has proven to be an essential protein, whose deletion leads to lethal defects in centrosome translocation and cytokinesis (Gunsalus et al., 1995), while in contrast, cofilin overexpression have been shown to increase cell movement. (Aizawa et al., 1996). In addition to the promotion of new filament polymerization at the leading edge, cofilin is known to promote filament branching by providing free barbed for nucleation via the ARP2/3 complex resulting in membrane protrusions from which invasion structures such as lamellopodia and filopodia are born. In parallel action, cofilin supports contractions at the rear end of the cell through focal depolymerization of F-actin and the regulation of actin myosin assembly,

by inhibiting binding of myosin II to F-actin. These actin/myosin regulated cell contractions at the rear end of the cell are critical to pull the cell forward finalizing the cell motility cycle. The multiple roles of this single protein in the control of cytoskeletal actin dynamics makes cofilin a critical modulator of processes as diverse as biological embryonic development and pathologic tumor development and metastasis.

Regulation of Cofilin Activity

The function of cofilin is temporally and spatially regulated via three types of mechanisms, dephosphorylation of cofilin at Serine 3 residue; the release of cofilin from PtdIns (4, 5)P₂ and the release of cofilin from cortactin (Mizuno, 2012). Although, the binding of cofilin to PtdIns (4,5)P₂ inactivates cofilin by sequestering the protein at the plasma membrane and the release from PtdIns(4,5)P₂ via its hydrolysis by phospholipase C (PLC) renders cofilin available for the initiation of actin severing, the activity status of cofilin is directly regulated via phosphorylation and dephosphorylation of a single serine residue at position 3 (Arber, 1998). Cortactin binding comprises an alternative mechanism to negatively regulate cofilin's activity, however this mechanism is known to be specific to the development of invasion targeted protrusions such as invadopodia (Weaver et al., 2006, Buccione et al., 2009, Linder et al., 2011). Phosphorylation of cofilin on Ser3 inhibits its binding to G actin (monomeric actin) and F-actin (filamentous actin) and severing of the actin filament at the leading edge of migrating cells. Several studies have documented differences in the cellular distribution of phosphorylated and

non-phosphorylated cofilin; being non-phosphorylated cofilin present in motile and invasive protrusions such as lamellopodia and filopodia of epithelial cells and phosphorylated cofilin uniformly distributed in the cytoplasm with the exception of the leading edge (Bravo et al., 2013). Four kinases have the ability to phosphorylate cofilin *in vivo*, LIM kinase -1, LIM kinase -2, (LIMK1, LIMK2), TES kinase 1 and TES kinase 2 (TESK1, TSK2) (Moriyama et al., 1996, Arber et al., 1998). LIMKs are known to be the dominant kinases involved in the regulation of actin dynamics, while TESKs are known to be involved in the process of focal adhesion via integrins signaling (Mizuno et al. 2012). The Rho-associated, coiled-coil containing protein kinase 1 (ROCK1), is responsible for LIMK-2 phosphorylation and activation. LIMK1 and 2 are activated via phosphorylation at Thr508 and Thr505 by several types of kinases including ROCK, PAK1, PAK2, PAK4, MRCK α and MAPK-activated protein kinase 2. ROCK1, phosphorylates and activates LIMK-2 in response to extracellular stimuli such as epidermal growth factor (EGF), leading to cytoskeletal reorganization via the activation of the RhoA/ROCK1 signaling pathway (Figure 1.8). Cofilin dephosphorylation at Ser3 and reactivation is performed mainly by Slingshot (SSH) phosphatase and chronopin (CIN) a phosphatase recently found to be specific for cofilin dephosphorylation (Niwa et al., 2002, Mizuno et al., 2012). Actin polymerization regulated by cofilin dephosphorylation/activation is a convergence point in the intracellular signaling network through which extracellular stimuli impact actin cytoskeleton, invasion and apoptosis. Alterations in cofilin and its signaling modifiers have been reported in invasive breast and ovarian cancer (Nishimura et al., 2010).

Figure 1.7 Cofilin Severing Activity. The binding of cofilin to the ADP- actin filament induces stress to the actin filament, changes the twist of the actin filament promoting severing and disassembly. Cofilin severing activity on already existing filaments allowed free barbed ends for the rapid addition of new actin monomers resulting in the rapid growth of filaments and filament branching. The ARP2/3 protein complex serve as nucleation sites for new actin filaments while profilin incorporates actin monomers at the growing end of new actin filaments.

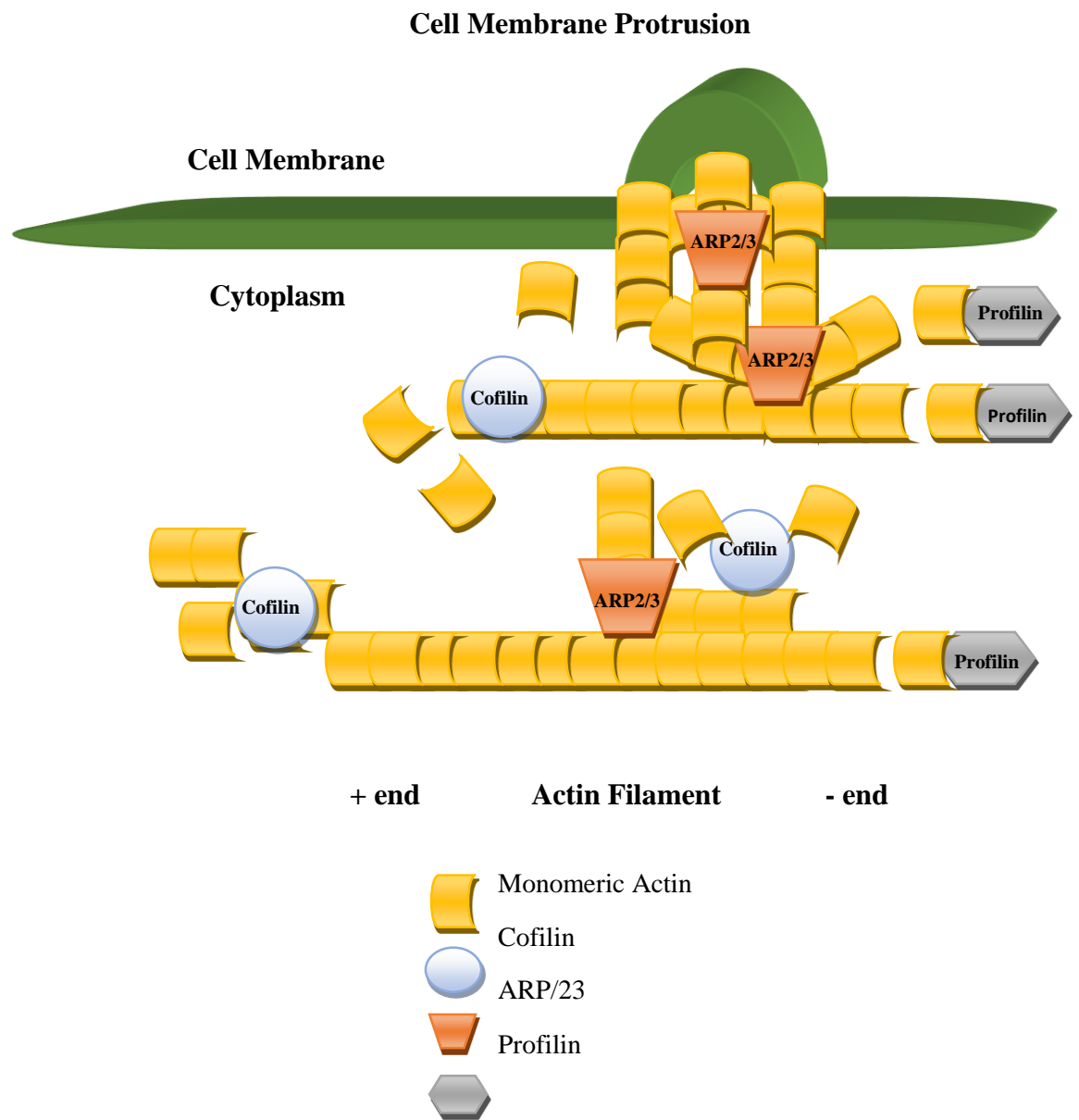


Figure 1.7 Cofilin Severing Activity

Figure 1.8 Regulation of Cofilin Activity/Function. The Rho-associated, coiled-coil containing protein kinase 1 (ROCK1), is responsible for LIMK-2 phosphorylation and activation in response to extracellular stimuli, leading to cytoskeletal reorganization via the activation of the RhoA/ROCK1 signaling pathway. Phosphorylation of cofilin by LIMK-2 at a specific Serine 3 residue, inhibits cofilin actin severing activity.

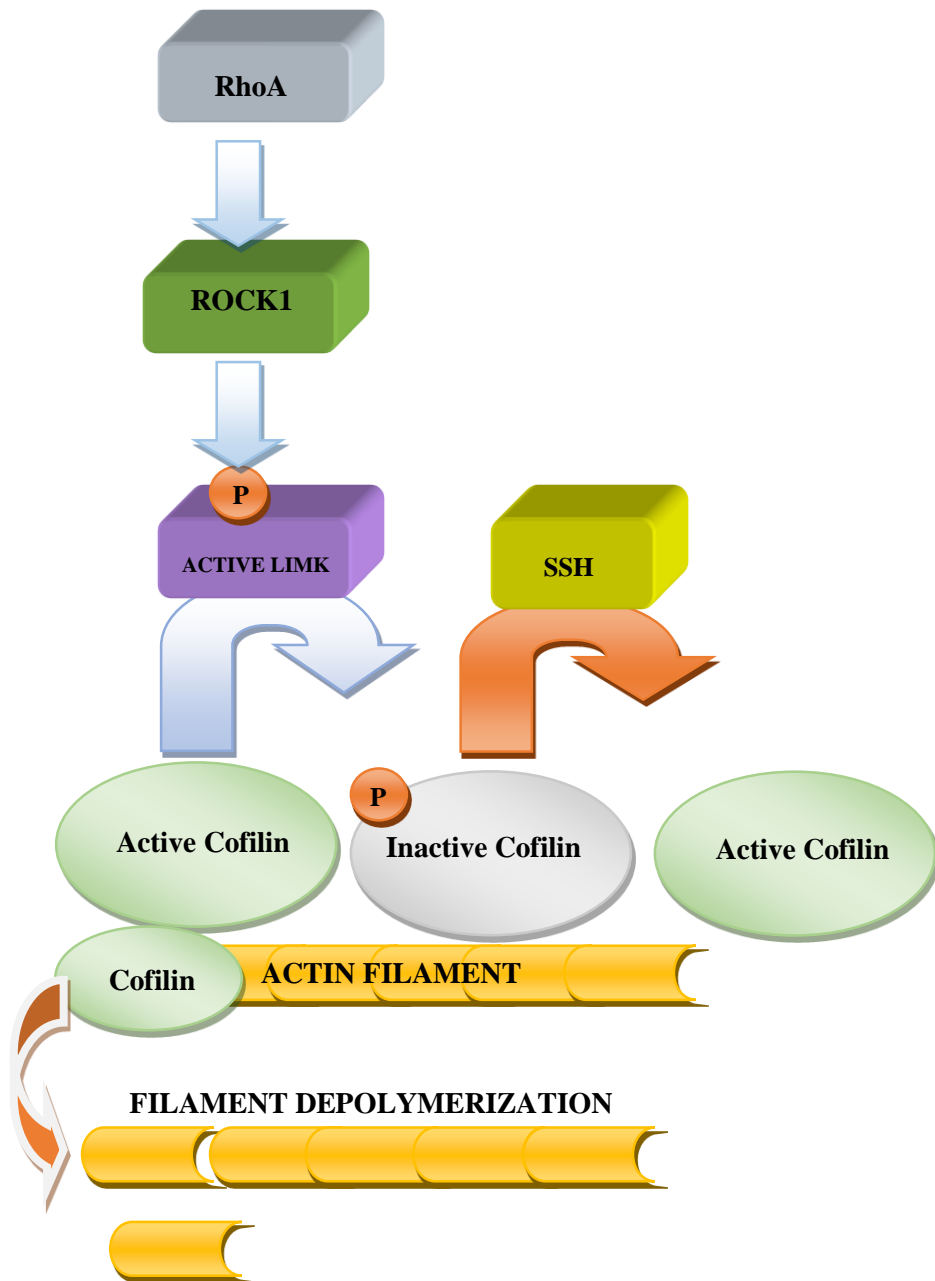


Figure 1.8 Regulation of Cofilin Activity/Function

Cofilin in Cancer

The critical role of cofilin in the modulation of cell movement and invasion lead to studies correlating cofilin activity and cancer. Growing evidence revealed the overexpression of cofilin protein, as well as genes encoding key regulators of the cofilin signaling pathways in different types of cancers like breast, lung, pancreatic and ovarian cancer (Sinha et al. 1999, Nishimura et al. 2010). As an example cofilin was found to be overexpressed in the highly invasive C6 rat glioblastoma cell line, A549 human lung cancer cells and human pancreatic cancer cells (Sinha et al., 1999, Gunnensen et al., 2000, Keshamouni et al., 2006). Studies in mammary carcinoma cells involving the expression of a constitutively active LIMK1 that increases the amount of phospho-cofilin *in vivo* demonstrated the inhibition of actin polymerization and motility in response to EGF, confirming the impact of alterations in key regulators of cofilin signaling on cancer cell movement (Zebda et al., 2000). Additional studies confirm this phenomenon in various cell lines since the overexpression of LIMK1 lead to the inhibition of cell movement in neuroblastoma cell lines and Ras-transformed fibroblasts (Sahai et al., 2001, Myer et al. 2005). In contrast, a dominant-negative LIMK1 results in increased movement in neuroblastoma cell lines. Moreover, *in vivo* studies demonstrated the metastatic potential of orthotopic mammary tumor cell inoculation to be directly related to the functional output of the cofilin pathway (Wang et al. 2006). As an example, tumors harboring suppressed cofilin activity due to cells in which cofilin pathway activity due to LIMK1 overexpression exhibited decreased invasion and metastasis and were associated with increased survival; in contrast, tumors derived from cells with increased cofilin pathway

activity (LIMK dominant negative) showed increased invasion and metastasis and were associated with decreased survival (Wang et al. 2006). This evidence suggests a link between tumor cell behavior and expression patterns of genes encoding cofilin and key regulators of the cofilin pathway and provided a new insight into the interaction of cytoskeletal modulators with factors in the tumor microenvironment. However the precise mechanisms via which cofilin modulates cytoskeletal remodeling in response to stimuli from the tumor microenvironment, and how those signals are interpreted by cancer cells are still not well understood.

CHAPTER II

RATIONALE AND SPECIFIC AIMS

Rationale

Metastasis is the cause of 95% of cancer-related deaths. Cancer metastasis is mediated by cellular interactions in response to signals from the tumor microenvironment affecting cytoskeletal actin polymerization and depolymerization leading to the modulation of cell adhesion, migration and invasion. TGF- β is known to be highly expressed in the prostate tumor microenvironment. This growth factor is the quintessential negative growth factor via its ability to inhibit proliferation and induce apoptosis. In addition to TGF- β 's role as a tumor suppressor this growth factor is known to switch roles and become a tumor promoter at late stages of the disease via the promotion of cell invasion, angiogenesis and the modulation of cell adhesion and migration.

The actin binding protein cofilin is directly functionally responsible for the remodeling of actin filaments and filopodia formation toward cytoskeletal reorganization, ultimately driving cell motility. Previously, evidence from our group, demonstrated that the small actin binding protein and main regulator of cytoskeleton actin dynamics cofilin to be a SMAD independent effector of TGF- β 's. Understanding how cancer cells interpret TGF- β signals from the microenvironment, is significant for defining the mechanism via which TGF- β function is switched from a growth suppressor to a metastasis promoter.

Thus, I hypothesized that there is an association between cofilin and prostate cancer metastatic progression that may be of significant predictive and targeting value in metastasis. Hence, the main goals of my work (presented on this thesis) are the following: 1) to dissect the molecular mechanisms by which the actin binding protein cofilin modulates actin cytoskeleton remodeling in prostate cancer cells , towards cell movement, attachment and invasion towards prostate cancer metastasis, 2) to study cofilin as a SMAD independent effector of TGF- β and the effect of the tumor microenvironment in cytoskeletal remodeling, cell migration, adhesion and invasion in prostate cancer cells.

Specific Aims

Specific Aim 1: Determine the consequences of a mutation in cofilin phosphorylation (regulation) site Ser 3 in cofilin expression and phosphorylation and the molecular mechanisms by which TGF- β modulates cofilin activity in prostate cancer (PC-3) cells. To gain a mechanistic insight into the role of cofilin in directing TGF- β responses towards cytoskeletal remodeling. This study will pursue the identification of molecular players directly involved in cofilin modulation by TGF- β .

Specific Aim 2: Determine the biological function of a mutation at cofilin phosphorylation site Ser3 in prostate cancer (PC-3) cells. The effect of a mutation in cofilin phosphorylation site on prostate cancer cell migration, adhesion and filopodia formation is assessed on WTCFL and S3ACFL PC-3 mutants mimicking the

constitutively active form of cofilin; as well as in S3DCFL mutants mimicking the constitutively inactive form. The impact of S3ACFL mutation on prostate cancer cell invasion in the presence of CAFs will allow the interrogation of the function of cofilin phosphorylation (regulation) in prostate cancer cell migration, invasion and adhesion in the context of the tumor microenvironment. This study will investigate the role of the tumor microenvironment in the regulation of biological processes targeting the actin cytoskeleton affecting prostate cancer progression to metastasis.

Specific Aim 3: Determine the *in vivo* consequences of a mutation on cofilin phosphorylation site and its effect on prostate cancer progression to metastasis. To determine the *in vivo* role of cofilin in prostate cancer metastasis, an experimental metastasis assay is performed. This study will extend and confirm the *in vitro* studies described in Specific Aim 2 and will enable the identification of the impact of cofilin S3A mutation on the process of prostate cancer in an *in vivo* physiologically relevant setting.

Specific Aim 4: Determine the value of cofilin expression in prostate cancer metastasis. These experiments will evaluate the expression profile of total and phosphorylated cofilin in the transgenic adenocarcinoma of the mouse model (TRAMP) and in primary and metastatic prostate tumors from the same patient cohort.

CHAPTER III

EXPERIMENTAL APPROACH

Cell Lines

The androgen independent human prostate cancer cell line PC-3, originally established from a patient with bone metastasis and known to be highly tumorigenic and metastatic in xenograft models (Kaighn et al. 1979) was obtained from the American Type Culture Collection (Manassas, VA). PC-3 cells were cultured in RPMI 1640 medium containing 2.05 mM L-glutamine, 2 g/liter sodium bicarbonate and 2 g/litter glucose (Invitrogen, Carlsbad, CA) together with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C under a humidified atmosphere of 5% CO₂. To test our findings in a secondary androgen responsive prostate cancer cell line we used the androgen sensitive prostate cancer cell line LNCaP overexpressing the TGF-β receptor II (LNCaPTRII) previously generated in our laboratory (Guo et al. 1999). LNCaPTRII cells were cultured under the same conditions as the PC-3 cell line. Primary cultures of human prostate cancer associated fibroblasts (CAFs) were established from radical prostatectomy specimens (from prostate cancer patients). Five distinct primary cultures of human prostate cancer associated fibroblasts from prostatectomy specimens were generated. Characteristics of CAFs (PCa-Str2-6) were analyzed by western blotting for the expression of AR, prostate specific membrane antigen, (PSMA), epithelial markers cytokeratin-18 (CK-18) and c-Met and stromal marker α-smooth muscle actin in a comparative analysis with human prostate cancer epithelial cell lines (LNCaP, PC-3,

DU145). CAFs were maintained in SCBM CC 3204 (Lonza, Walkersville, MD) at 37°C under a humidified atmosphere of 5% CO₂.

Isolation of Prostate Cancer Associated Fibroblasts (CAFs)

Human prostate tumor tissue was severed by a scalpel in multiple small fragments in SCGM (SCBM+5%FBS) medium in a tissue culture plate. Culture plate was incubated undisturbed for 5-7 days (medium was refreshed). After 1-2 weeks, plates were washed with PBS, trypsinized for ~2 minutes to detach only stromal cells. Floating cells were collected, centrifuged, and resuspended and cultured in SCGM, incubated for 3 days, trypsinized again. The process was repeated in T175 flask. Cells were harvested and frozen in multiple vials and stored in liquid nitrogen. Vials were cultured in SCGM and tested for cyto differentiation markers by western blotting

Transfections

The S3A cofilin mutant prostate cancer cell line was generated by site directed mutagenesis in PC-3 cells. A point mutation targeting Ser 3 phosphorylation site was induced by PCR. To mimic a dephosphorylated (constitutively active) form of cofilin (S3ACFL mutants), a substitution of a Serine on position 3 to Alanine was generated. WT and S3ACFL mutant forms of cofilin were introduced into PC-3 cells via stable transfection. S3D cofilin mutation, mimicking the constitutive phosphorylated (inactive) form, was introduced in PC-3 cells via transient transfection. Expression vectors for S3D cofilin were generous gifts from Dr. Sergei Krupenko, (MUSC, Charleston, SC). Cells were transfected with pXJN-HA/cofilin vector DNA using the Effectene Transfection

Reagent (QIAGEN 301425, Hilden, Germany). Cofilin expression was silenced in PC-3 cells by using the siRNA sequence targeting cofilin codons 64-84. A siRNA containing a two single-nucleotide mutation of cofilin sequence (C71G and A73U) was used as control.

Cell Viability

To assess cell viability we used the MTT assay. Cells (10^4 /well) were seeded into 96-well plates and incubated in growth medium (24 hrs). After incubation with the MTT solution for 4 hrs, and after dissolving formazan crystal absorbance was read at 490nm (Bio-RAD 680, USA) and the colorimetric reaction product was quantitated spectrophotometrically.

Migration Assay

The migration ability of WTCFL and mutants S3ACFL (active form of cofilin), S3DCFL (inactive form of cofilin) prostate cancer cells was analyzed via wound assay. Cells (10^4 cells/well) were seeded in 6 well multiwell plates, allowed to grow to 60-70% density of cell monolayer and a wound was induced using a pipet tip. After wounding, cells were exposed to TGF- β (Recombinant Human TGF- β 1, R&D Systems, MN, USA) (5ng/ml; 24hrs) in the presence or absence of TGF- β (Human LAP TGF- β 1 Antibody, R&D Systems, MN USA) neutralizing antibody. The number of migrating cells was counted in three different fields, under microscopic examination.

Adhesion Assay

A subconfluent cell culture of wild type and mutants S3ACFL, S3DCFL PC-3 cells was treated with TGF- β (5ng/ml, 24 hrs) at 37°C. Cells (40,000 cells/well) were seeded in 6-well plates pre-coated with fibronectin (0-8 μ g/ml). After 30 min incubation (at 37°C) non adherent cells were removed and adherent cells were fixed with ice cold methanol (5min). The number of cells attached was evaluated in three different fields under microscopic examination.

Fibronectin Coating

Fibronectin, Humanplasma (1mg) (Calbiochem) was thaw by placing the vial in a 37°C water bath undisturbed until completely thawed. Pre-warmed, sterile, serum free 16140 RPMI (Invitrogen) culture medium was added to the solution to a final volume of 20ml to yield a fibronectin work solution of 50 μ g/ml. The fibronectin work solution (1ml) was added to each well of a 6 multiwell plate, gently swirling the solution to evenly coat the bottom of the well. The plates were incubated at room temperature for 45 minutes to allow binding of the fibronectin to the surface of the well. Finally fibronectin work solution was removed by tilting the plate and gently aspirating with a sterile pipet.

Evaluation of Cell Invasion

(a) **Matrigel Invasion Assay:** The invasion potential was evaluated using a Biocat Matrigel Transwell Chamber (Beckon Dickinson, Franklin Lakes, NJ). Cells were seeded into the upper chamber of a transwell insert pre-coated with matrigel in serum-free medium (50,000 cells/well). After 24hrs non-invading cells were removed from the

upper chamber and invading cells were stained with Diff-Quick Solution (IMEB Inc., San Marcos, CA).

(b) Matrigel Cell Tracking: Human prostate cancer-associated fibroblasts (CAFs) and PC-3 prostate cancer cells were independently grown in medium containing SCBM or RPMI respectively. SCBM medium containing CellTracker™Green CMFDA dye (5μM) (Invitrogen USA) and 1640 RPMI medium containing CellTracker™ Orange CMTML (Invitrogen USA), were added to CAFs and PC-3 cells, respectively (45mins). The CellTracker™ Green CMFDA labeled cell suspensions of CAFs were placed into the inner circle of underside membrane of a Biocat Matrigel Transwell Chamber precoated with matrigel. Inserts were placed in 12-wells in Biocat Matrigel Transwell Chambers in the absence or presence of TGF-β ligand (5ng/ml). CellTracker™ Orange CMTML labeled WT and S3ACFL PC-3 cells were seeded in the upper chamber and after 24hrs invading cells were detected using an epifluorescence Nikon Eclipse E600 microscope (Nikon, Melville, NY).

In-vitro Co-culture Assay

Human prostate cancer associated fibroblasts (CAFs) were grown in the inner membrane circle of Biocat Matrigel Transwell Chamber inserts and after 24hrs, inserts were transferred in Biocat Matrigel Transwell Chambers in absence/presence of TGF-β neutralizing antibody 5ng/ml. Prostate cancer epithelial cells were seeded into the upper chamber and after co-culturing for 24hrs invading cells are stained with Diff-Quick Solution (IMEB Inc, San Marcos, CA) and visualized under light microscopy.

Western Blot Analysis

Cell pellets and lung tissue were lysed in RIPA buffer (50mM Tris-HCl, pH7.4, 1%NP40, 0.25% Na-deoxycholate, 150 mM NaCl, 1mM EDTA, 1mM PMSF, (Sigma P8340 protease inhibitor). Protein (30µg) as a cell lysate or tissue homogenate was fractioned by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on gradient gels and transferred to Immuno-Blot PVDF membranes. The membrane was blocked with 0.05% Tween-20 and 5% defatted milk (1hr at room temperature) followed by incubation to the respective primary antibodies in the same buffer overnight at 4°C with gentle shaking. After 24 hrs the immunoblot was developed with a secondary antibody conjugated to horseradish peroxidase (1hr at room temperature) and proteins bands were detected using the ECL plus Detection System (GE, Amersham, UK). The chemiluminescent image was captured by autoradiography. The antibodies used were: anti-cofilin (1:10,000) (Sigma Aldrich, St. Louis, MO), phospho-cofilin (Ser 3) (1:10,000); anti-LIMK-2(1:1000) (Cell Signaling, Danvers, MA) and (GFP1:100) (Santa Cruz Biotechnology, CA).

Immunoprecipitation Assay

For the immunoprecipitation experiments, PC-3 cells were transfected with Flag-tagged WTCFL, S3ACFL, and T25A CFL, and cells were grown in CSS medium for 24hrs. Cells were subsequently treated with TGFβ1 (for 6hrs) in the absence or presence of MEK inhibitor PD98095. Whole cell lysates were subjected to immunoprecipitation with the anti-Flag antibody, and Western blots with the specific antibodies as follows:

Cells were lysed by sonication in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, and 1 mM EDTA) supplemented with protease inhibitor cocktail, 1 mM PMSF and 10 units/ml of DNase I (New England Biolabs). After centrifugation at $21,000 \times g$ for 12 min at 4°C, the supernatants/clarified lysates were isolated and their protein concentrations measured. Aliquots of the clarified lysates (~600 ug of protein each) were then pre-cleared with Protein G Plus/Protein Agarose beads (Calbiochem) and 1 ug of normal mouse IgG (Santa Cruz) for 1 h, then incubated with anti-acetylated lysine antibody (Cell Signaling) and Protein G Plus/Protein (30 μ l), 18 h at 4°C. After collection by centrifugation and removal of supernatant, the beads were then washed three times with RIPA buffer supplemented with protease cocktail inhibitors, 1 mM PMSF and 200 ug/ml ethidium bromide. After removal of the final wash, equal portions of RIPA and 2X SDS sample buffer were added to the beads and immunoprecipitated proteins were released by heating at 95°C for 5 min. Equal volumes of each sample were resolved by SDS-PAGE (8%).

Immunofluorescence Staining

Cells (7×10^4 cells/well) were seeded in 6-well plates containing a glass cover slit and exposed to TGF- β (5ng/ml, 24hrs). Cells were fixed in cover slit by adding ice cold methanol-free formaldehyde (15mins), followed by two washes with PBS and permeabilization with 2ml of Triton X-100 (0.1% v/v) (5mins). After permeabilization cover slits were exposed to Rhodamine Phalloidin for fluorescent staining of filamentous actin (Invitrogen). Cofilin expression was detected by blocking the coverslit on goat serum buffer for 1hr followed by incubation with primary rabbit anti-cofilin antibody

(1:10,000) for 24hrs at 4°C (Sigma Aldrich, St. Louis, MO). Cover slits were subsequently removed, washed with PBS and incubated with Alexa Fluor 488 (Invitrogen) (1hr). Images were processed using a fluorescence Nikon Eclipse E600 microscope (Nikon, Melville, NY).

Experimental Metastasis Assay

The metastatic potential of WTCFL and S3ACFL mutant PC-3 cells was examined *in vivo* by the tail vein injection-experimental metastasis assay. Male nude mice (6-wks old) (Harlan Laboratories Inc., Indianapolis, IN) were maintained in sterile cages in pathogen-free environment. Animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee. GFP labeled WTCFL and S3ACFL PC-3 10^6 cells were injected into the tail vein of mice (n=6/cell line). At 4-wks post-inoculation, lungs were excised and metastatic lesions to the lungs were counted under a dissection microscope.

Wild type and mutant S3ACFL PC-3 cells were cultured in T-75 flasks. Cells were harvested by trypsinization and cell suspensions were submitted to centrifugation (1,000 RPM, 5 minutes). After centrifugation the supernatant was discarded and 1ml of fresh growth medium was added. A cell suspension containing 10^6 cells per ml was prepared as described above. A total of 1ml of cell suspension was injected into the lateral saphenous tail vein as followed:

- (a) Mice were restrained via the injection of the anesthetic (Ketamine, conc. 100mg/ml) (0.1ml per 10 gm of body weight).

- (b) Periferal vasodilatation of tail vein was induced by submerging the mouse tail into a glass beaker containing warm water (5 minutes).
- (c) The injection area was disinfected with ethanol swipes and tourniquet-like pressure was applied to the bottom portion of the tail.
- (d) A 28 ½ gauge needle containing 1ml of cell suspension was injected into the vein at a slight angle.

After injection needle was removed and pressure was applied at injection site.

Preparation of Lung Tissue Homogenates

Lung tissue homogenates were prepared as followed: Right and left lungs were transferred into a Dounce and Potter-Elvehjem homogenizer containing chilled RIPA homogenization buffer (50mM Tris-HCL, pH 7.4, 150mM NaCl, 1mM PMSF, 1mM EDTA, µg/ml aprotinin, 5 µg/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS). Protease and phosphatase inhibitors were added and samples were subjected to freeze-thawing (2cycles) and centrifugated (at 18,300xg, 10 min at 4°C). Supernatants were subjected to Western Blot analysis.

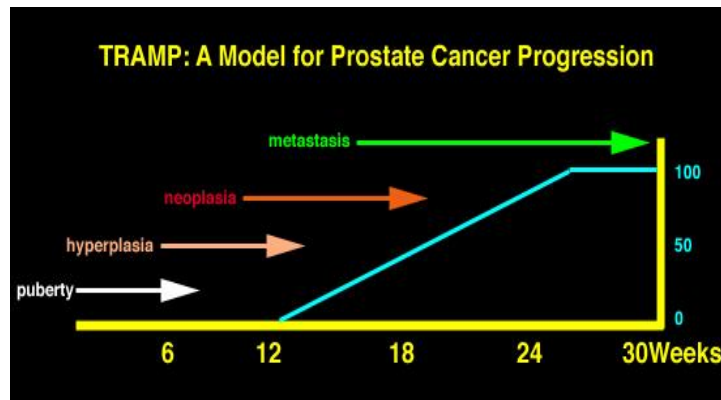
Immunostaining Analysis of Paraffin Embedded Human Prostate Specimens

Human Prostate Specimens: Formalin fixed paraffin embedded specimens of human local and metastatic prostate cancer were obtained from the University of Kentucky Cancer Center Tissue Biobank with Institutional Review Board Approval. Sections (4µm) were affixed to glass slides, deparafinized and rehydrated. Total cofilin and p-cofilin expression was

detected using the following antibodies: (C8736) Anti - cofilin from Sigma Aldrich (St. Louis, MO), (3311) Phospho – cofilin (Ser 3) from Cell Signaling Technology (Danvers, MA). Palladin expression was detected using (10853-1-AP) PALLD palladin antibody from Proteintech Group. E- Cadherin expression was detected using (24E10) E-Cadherin rabbit Ab from Cell signaling Technology (Danvers, MA). Sections were incubated with the corresponding primary antibodies (1:50 to 1:100) overnight at 4°C). After two washes with 1x TBS - 0.1% Triton – X slide sections were incubated with Millipore (21537) IHC select immunoperoxidase secondary detection system (1hr) followed by incubation with Millipore Streptavidin HRP (1hr at room temperature). Peroxidase activity was detected by applying Diaminobenzidine (DAB) and counterstained with hematoxylin. Slides were analyzed under a light microscope followed by pathological evaluation. Images were photographed at 40x magnification.

TRAMP Mouse Model: The transgenic adenocarcinoma mouse prostate (TRAMP) was used to investigate the correlation between cofilin and phosphorylated cofilin expression and prostate cancer progression. TRAMP is considered a suitable model of prostate tumorigenesis. As shown below, TRAMP transgenic male mice develop prostate cancer from prostatic intraepithelial neoplasia (PIN) into metastatic androgen independent carcinoma in a manner resembling the clinical progression of human prostate cancer patients. The TRAMP transgene is in the C57BL/6J genetic

background. TRAMP mice are transgenic mice that express SV40T/t antigen under the prostate specific rat probasin promoter. Tissue sections from (16, 20, 24, 28 wks) TRAMP prostate tumors of increasingly aggressive stage were subjected to immunohistochemical analysis as above.



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Figure 3.1: TRAMP Mouse Model of Prostate Cancer Progression

Statistical Analysis

Statistical analyses are performed with GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA). Data are presented as \pm Standard Error of the Mean (SEM). Numerical values are the mean of three independent experiments. Statistical evaluation of the data is performed using the Student *t* test and Two-way analysis of variance for multiple comparisons. Significant difference is defined at P value < 0.05.

CHAPTER IV

RESULTS

Cofilin Activity Directs TGF- β Mediated Actin Severing in Prostate Cancer Cells

Phosphorylation of cofilin (CFL) on Ser3 inhibits its binding to G actin (monomeric actin) and F-actin (filamentous actin) and severing of the actin filament at the leading edge of migrating cells. Constitutively active (dephosphorylated) forms of cofilin were generated in PC-3 prostate cancer cells by mutagenesis via substitution of a Serine on position 3 to Alanine (S3ACFL mutants) and a Threonine to Alanine at position 25 (T25ACFL mutants). Immunoprecipitation analysis of phosphorylated protein associations in response to TGF- β , revealed that the S3ACFL mutation specifically conferring cofilin dephosphorylation, promotes its association with actin (enhancing filament severing), while the T25ACFL mutation, (impairing Threonine phosphorylation) had no effect on the association of p-cofilin with actin (Figure 4.1). Moreover, the presence of MEK inhibitor (PD98059) abrogated the TGF- β mediated association between p-Erk and cofilin (Figure 4.1). Mutational activation/cofilin dephosphorylation (S3ACFL or T25ACFL), or loss of cofilin expression (shCFL) had no significant consequences on prostate cancer cell viability (Figure 4.2). The S3A cofilin mutation, as expected, abrogated its phosphorylation by LIMK-2, without affecting total cofilin expression (Figure 4.3 A); there was a compensatory upregulation of LIMK-2 levels in the S3ACFL PC-3 cells compared to wild type cofilin PC-3 (WTCFL) (Figure 4.3 A,

Panel A). Figure 4.3 panel B reveals the endogenous upregulation of downstream signaling effectors RhoA and ROCK1 induced by the introduction of cofilin mutations; both S3DCFL and S3ACFL cells exhibited a significant increase in protein expression for RhoA and ROCK1, compared to WTCFL cells. In response to TGF- β , there was a transient induction in phosphorylated cofilin within 3 to 6hrs, that was preceded by a significant increase in ROCK 1 and Rho A levels, in the WTCFL but not in the S3ACFL cells (Figures 4.3, panels C and D).

Figure 4.1 Specificity of S3A Active Cofilin Protein Associations in Response to TGF- β . PC-3 cells were transfected with Flag tagged WTCFL, S3ACFL, or T25ACFL, after growing in CSS medium (24hrs), cells were treated with TGF β 1 (5ng/ml) for 6hrs with or without PD98095. Cell lysates (50 μ g of protein) were subjected to immunoprecipitation with anti-Flag antibody, and subsequent Western blotting with the indicated antibodies. Actin and phosphorylated proteins p-Erk and p-cofilin show enhanced association with WCFL in response to TGF- β . S3A CFL mutation confers constitutive dephosphorylation and thus cofilin fails to undergo TGF- β -mediated interaction with actin. In comparison, the T25A CFL mutation impairs threonine phosphorylation, but has no effect on the phosphorylated cofilin-actin association in response to TGF- β .

Figure 4.1 Specificity of S3A Active Cofilin Protein Associations in Response to TGF- β .

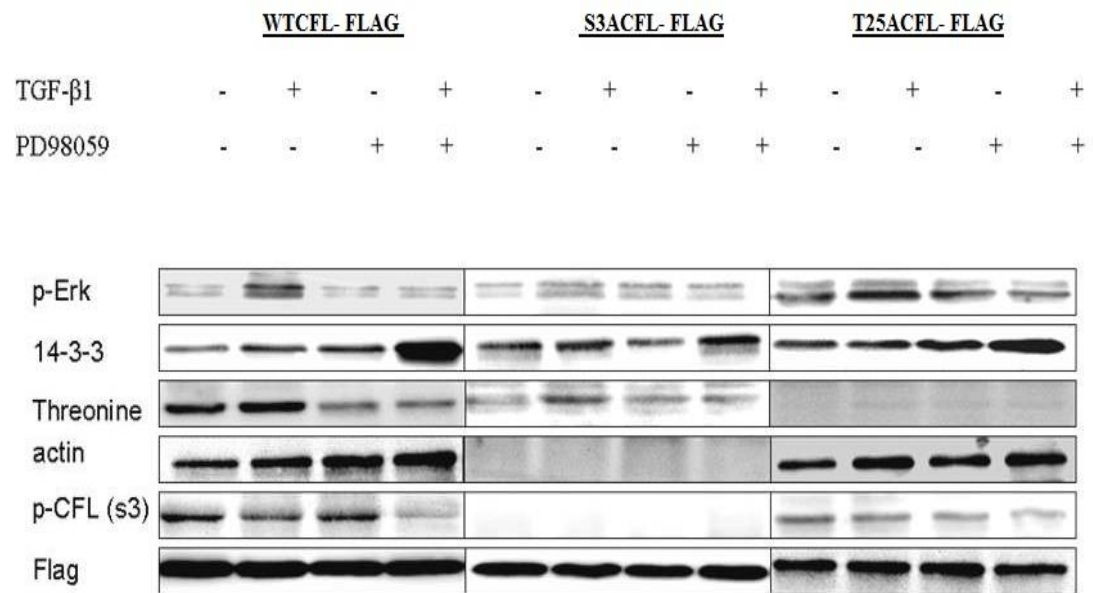
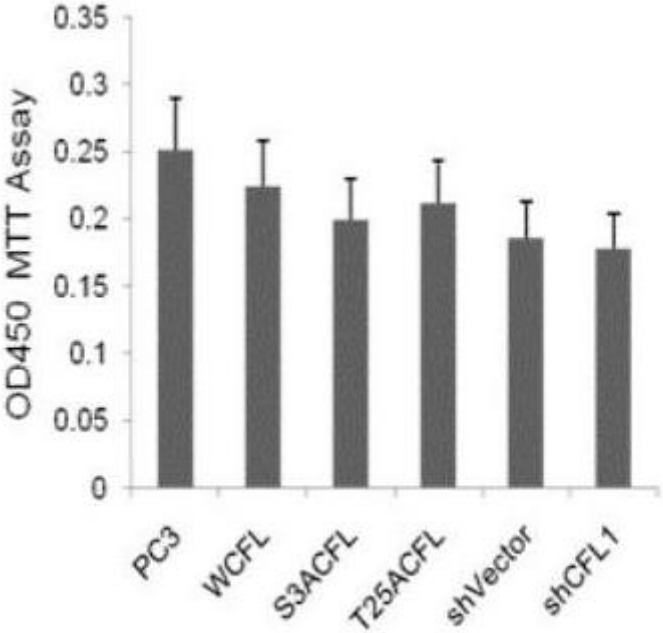


Figure 4.2 Effect of Cofilin Mutational Activation or Loss on Prostate Cancer Cell Death and Endogenous Cofilin Expression. Panels A: The effect of a mutation on cofilin phosphorylation site Ser3 or cofilin silencing was assessed by MTT assay. Neither shRNA silencing of cofilin expression or inducing a mutation on cofilin phosphorylation site has any effect on cell viability. Panel B: endogenous cofilin expression was assessed by Western blotting analysis in prostate cancer cell lines: PC-3, LNCaP, DU145 and C4-2 as well as in the breast cancer cell line MCF7. The androgen independent PC-3 and androgen sensitive LNCaP prostate cancer cell lines together with the breast cancer cell line MCF7 showed higher levels of endogenous cofilin compared to DU145 and C4-2 prostate cancer cell lines.

Figure 4.2 Effect of Cofilin Mutational Activation or Loss on Prostate Cancer Cell Death and Endogenous Cofilin Expression.

A.



B.

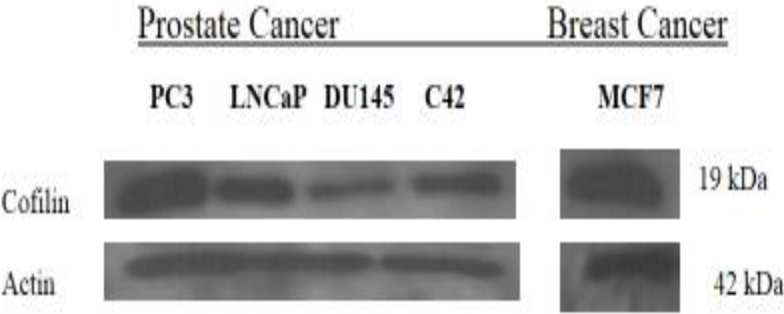
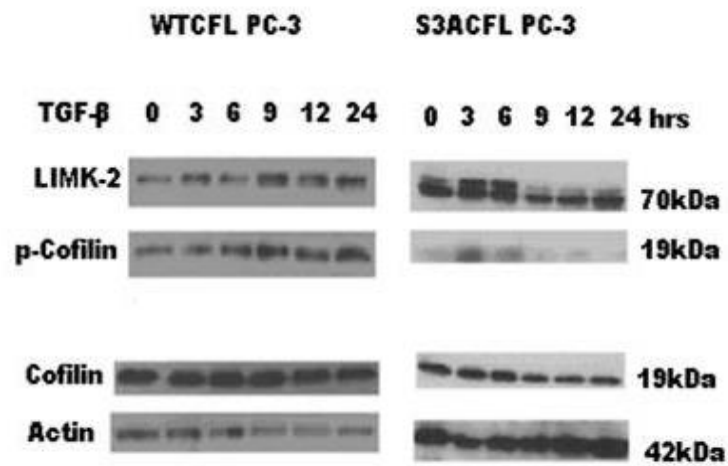


Figure 4.3 Effect of S3A Mutation on Cofilin Phosphorylation Events in PC-3 Cells.

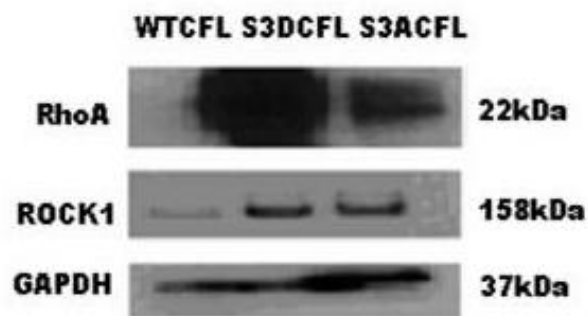
Effect of TGF- β on cofilin, p-cofilin and LIMK-2 protein expression in prostate cancer cells. Panel A, Upregulation of LIMK-2 protein in mutant S3ACFL PC-3 cells. Treatment with TGF- β (5ng/ml) increased LIMK-2 and p-cofilin expression in wild type PC-3 cells and decreased the expression of LIMK-2 in the S3ACFL cells. Panel B, Western blotting indicating elevated RhoA and ROCK1 protein in S3ACFL PC-3. Panels C and D, Treatment with TGF- β increased RhoA and ROCK1 levels in WTCFL cells and decreased expression of both proteins in S3ACFL cells. GAPDH was used as loading control. Molecular weights for individual proteins are shown on the right.

Figure 4.3 Effect of S3A Mutation on Cofilin Phosphorylation Events in PC-3 Cells.

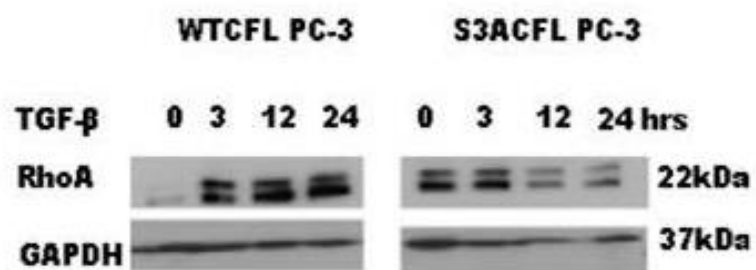
A.



B.



C.



D.



Active Cofilin Dictates Prostate Cancer Cell Migration and Invasion Responses to TGF- β

To determine the effect of a mutation in cofilin phosphorylation site on prostate cancer cell migration, we assessed the migration ability of WTCFL and S3ACFL mutants mimicking the constitutively active form of cofilin; as a functional control, S3DCFL mimicks the constitutively inactive form. The S3A cofilin mutation resulted in a significant increase in PC-3 cell migration (Figure 4.4). There was a decrease in cell migration in the mutant S3DCFL cells (Figure 4.4). As shown on Figure 4.5, treatment with TGF- β (24 hrs) led to a significant decrease in cell migration for WTCFL cells; functional blocking of TGF- β by the neutralizing antibody, restored migration capacity to control levels in WT cells (Figure 4.5). The impact of S3ACFL mutation on prostate cancer cell invasion was interrogated in the context of the tumor microenvironment. The quantitative data from the invasion assay indicate no significant difference in the invasion potential of S3ACFL cells compared to WTCFL PC-3 cells (Figure 4.6). The increase in invading cell number in response to exogenous TGF- β , was abrogated by the presence of the neutralizing antibody against TGF- β , in both WTCFL and S3ACFL cells ($p < 0.05$).

The reactive stroma contributes to prostate cancer progression through the cancer-associated fibroblasts that facilitate metastasis (Yang et al, 2007; Jung et al, 2013). To assess whether the effect of cofilin on prostate tumor cell invasion was TGF- β -dependent as mediated from surrounding tumor associated fibroblasts, prostate cancer cell invasion was evaluated in *in vitro* co-cultures. Fluorescent labeled PC-3 prostate cancer epithelial cells (red) were co-cultured with labeled human prostate cancer associated fibroblasts (green) in the upper chamber of a matrigel pre-coated transwell insert (24hrs) (Figure 4.7,

upper panel). As shown on Figure 4.7 (lower panel), only prostate tumor epithelial cells invaded the matrigel. There was no significant difference in cell invasion between WTCFL and S3ACFL cell lines (but there was a decrease in the S3D mutant cells). In the presence of human prostate cancer associated fibroblasts (CAFs) however, there was a significant increase in the number of tumor epithelial cells invading, for both WTCFL and S3ACFL PC-3 cells. The S3D CFL mutation (phosphorylation) had no effect on prostate cancer cell invasion regardless of TGF- β status (Figure 4.7) (lower panel). Simultaneous exposure to the TGF- β neutralizing antibody (5ng/ml) resulted in further significant increase in the S3ACFL invasion potential ($p < 0.004$), (while it reduced WTCFL PC-3 cell invasion), demonstrating that only active cofilin was able to functionally direct TGF- β signaling (secreted by the CAFs in co-cultures) towards enhanced invasive behavior while it further increased S3ACFL migration (lower panel).

Figure 4.4 Cofilin Activation Status Dictates Prostate Cancer Cell Migration.

Mutation in cofilin phosphorylation site Ser3 mimicking the constitutively active form of cofilin (S3ACFL) significantly increased prostate cancer cell migration compared to WTCFL ($p < 0.03$). Prostate cancer cells harboring the inactive form of cofilin (S3DCFL mutation) exhibited a significant reduction in migration capacity compared to S3ACFL cells ($p < 0.006$). Values shown are the number of migrating cells from two independent experiments performed in triplicate.

Figure 4.4 Cofilin Activation Status Dictates Prostate Cancer Cell Migration.

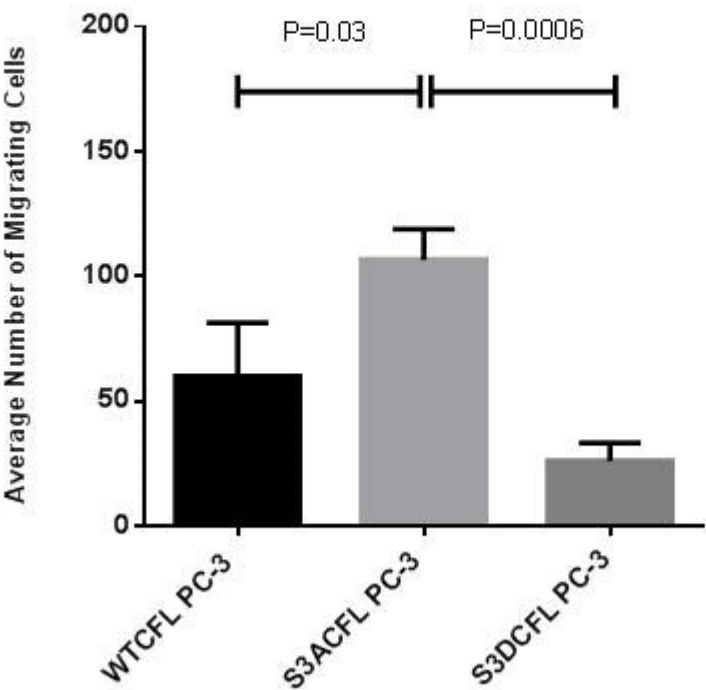
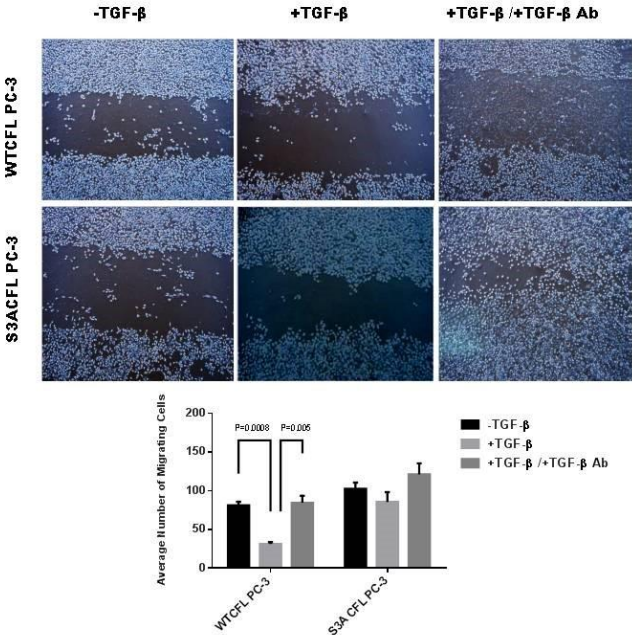


Figure 4.5 S3ACFL Mutation Enhances Prostate Cancer Cell Migration by passing TGF- β . Upper panel, representative images of increased cell migration ability for S3ACFL PC-3 cells compared to WTCFL cells (24hrs). TGF- β treatment significantly decreased WTCFL cell migration ($p < 0.0008$), but it had no significant effect in S3ACFL cells. Loss of TGF- β (in presence of neutralizing antibody) restored the WTCFL PC-3 cell migration capacity (to control levels), while it increased S3CFL mutant cell migration ($p = 0.005$). Cell (Lower panel) cell migration was also analyzed on the androgen sensitive prostate cancer cell line LNCaPTRII overexpressing TGF- β type II receptor. TGF- β treatment significantly decreased LNCaPTRII cell migration ($p = 0.0013$). Loss of TGF- β (in presence of neutralizing antibody) restored LNCaPTRII cell migration.

**Figure 4.5 S3ACFL Mutation Enhances Prostate Cancer Cell Migration by passing
TGF- β .**



LNCaPTRII

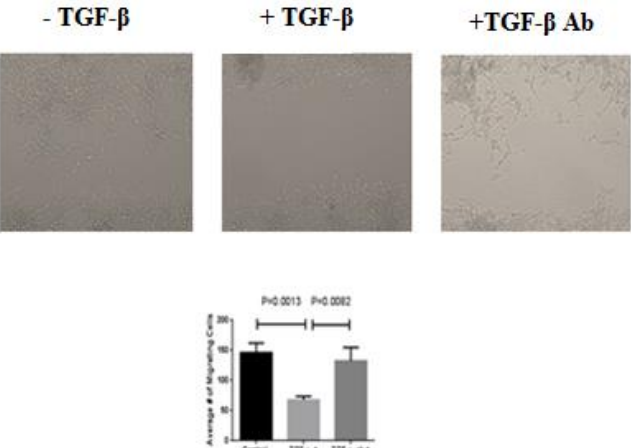


Figure 4.6 Cofilin Navigates Invasive Response to TGF- β . The invasive response of prostate cancer cells to TGF- β was assessed in the matrigel assay. The mutation on cofilin phosphorylation site had no significant effect on PC-3 cell invasion (black barographs). In response to exogenous TGF- β , there was an increase in WTCFL PC-3 cell invasion potential, but not in S3ACFL cells ($p=0.03$). Loss of TGF- β (by neutralizing antibody) led to a significant decrease in the invasion potential for both WTCFL and S3ACFL cells ($p=0.04$ and $p=0.004$, respectively). (Lower panel) cell invasion was also analyzed on the androgen sensitive prostate cancer cell line LNCaPTRII overexpressing TGF- β type II receptor. TGF- β treatment significantly increased LNCaPTRII cell invasion.

Figure 4.6 Cofilin Navigates Invasive Response to TGF- β .

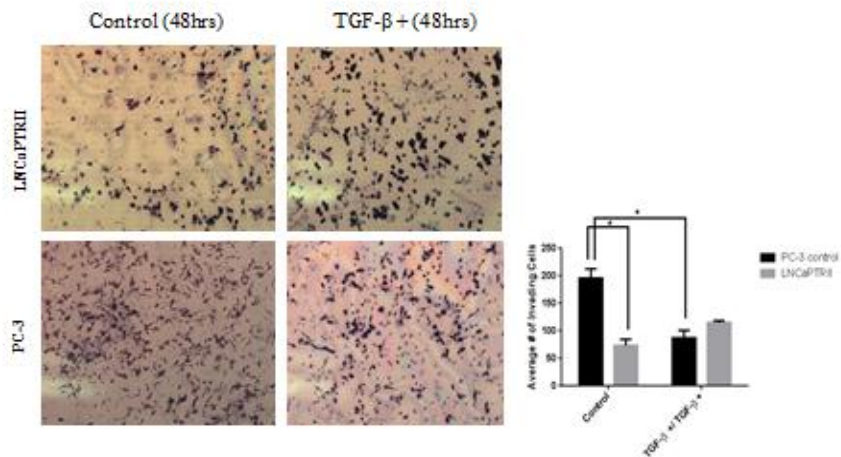
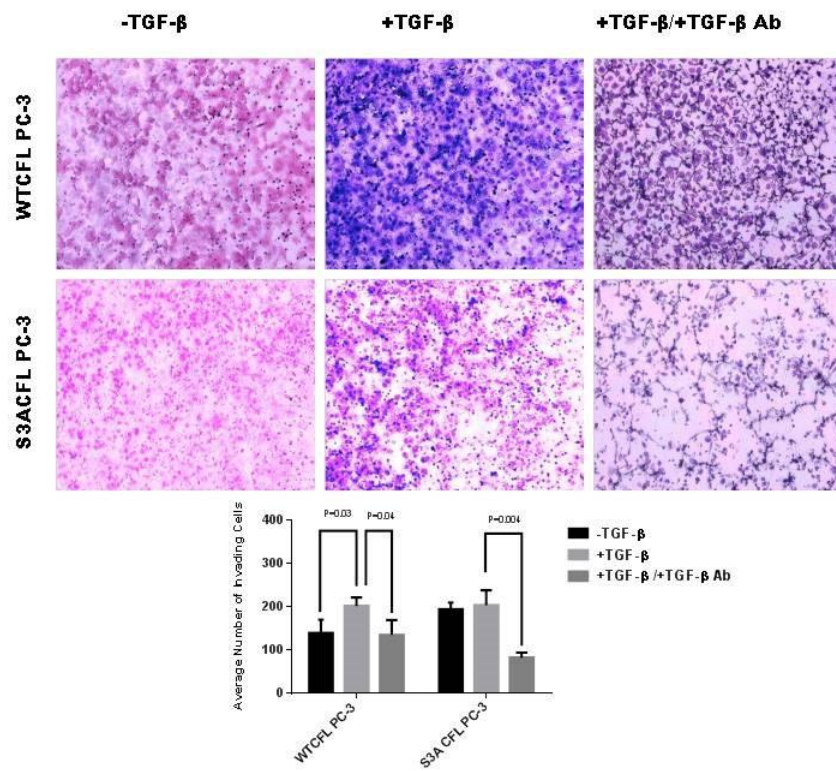
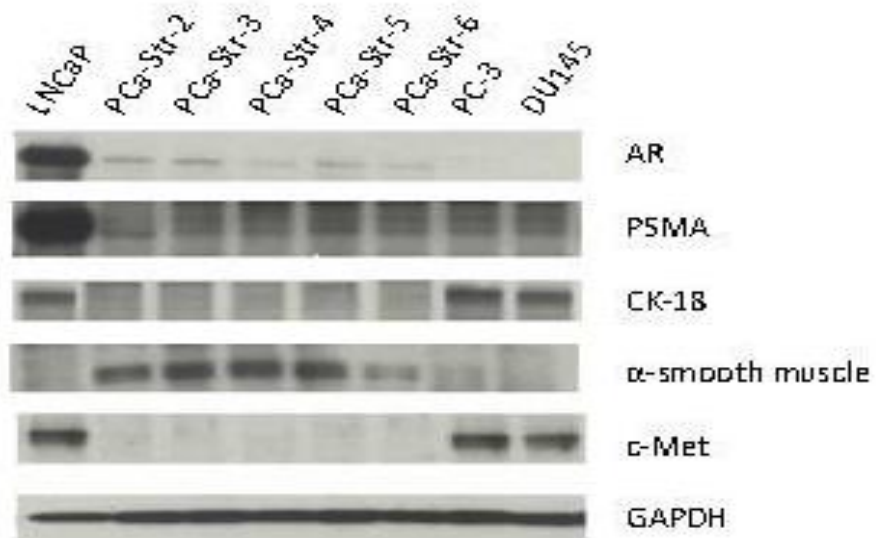


Figure 4.7 Co-Culture of S3ACFL PC-3 cells with Human CAFs Markedly Enhanced Prostate Cancer Cell Invasion. Panel A .Characteristics of 5 different prostate cancer associated fibroblasts CAFs were analyzed by Western blotting (PCa-Str2-6) in a comparative analysis with human prostate cancer epithelial cell lines (LNCaP, PC-3, DU145) for the expression of AR, prostate specific membrane antigen, (PSMA), cytokeratin-18 (CK-18), α -smooth muscle actin and c-Met. As shown in lanes 2-6, only the cancer associated fibroblasts were positive for the expression of the stromal marker, α -smooth muscle actin whether only prostate cancer cells were positive for the epithelial markers CK-18 and c-Met. Panel B, representative image of fluorescent labeled WTCFL PC-3 prostate cancer cells (red) and human cancer associated fibroblasts (green) co-culture (1:1) (48hrs). Panel C, matrigel invasion in co-cultures of WTCFL, S3ACFL and S3DCFL with CAFs in the presence or absence of a neutralizing TGF- β antibody. Quantitative assessment of invading cells indicates that only active cofilin (S3A mutation) directs a further increase in TGF- β mediated cell invasion (derived from CAFs). In WTCFL PC-3/CAF, and S3ACFL PC-3/CAF co-cultures after 24hrs, CAFs significantly increased prostate cancer cell invasion for both WTCFL and S3ACFL cells ($p=0.004$ and $p=0.007$) (lower panel lanes 1 and 2). Continuous secretion of TGF- β by the reactive microenvironment (in presence of TGF- β neutralizing antibody), induced a further increase in the number of invading S3ACFL cells ($p=0.008$), while it decreased WTCFL cell invasion. Values are the average from two independent experiments in triplicate.

Figure 4.7 Co-Culture of S3ACFL PC-3 cells with Human CAFs Markedly Enhanced Prostate Cancer Cell Invasion.

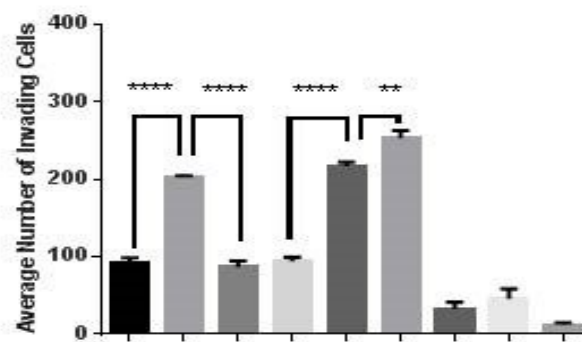
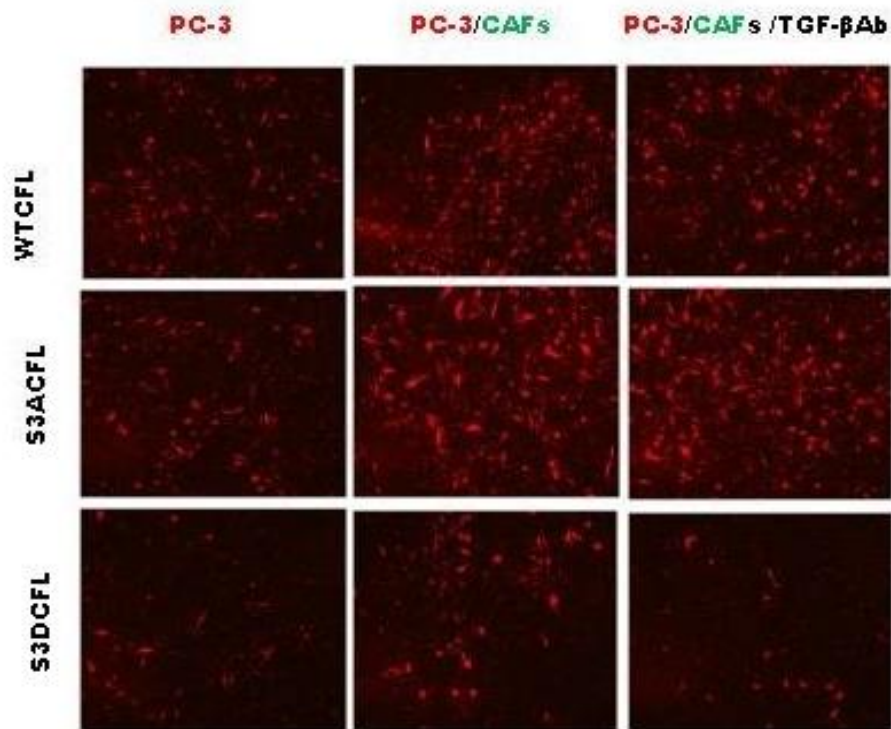
A.



B.



C.



| | | | | | | | | | |
|-----------------|---|---|---|---|---|---|---|---|---|
| WTCFL | + | + | + | - | - | - | - | - | - |
| S3ACFL | - | - | - | + | + | + | - | - | - |
| S3DCFL | - | - | - | - | - | - | + | + | + |
| CAFs | - | + | + | - | + | + | - | + | + |
| TGF-β ab | - | - | + | - | - | + | - | - | + |

Cofilin Mediates Prostate Cancer Cell Adhesion via Cytoskeletal Remodeling

Cell adhesion is directly dependent on cofilin activity and cytoskeletal actin since depolymerization and polymerization of new actin filaments is required for filopodia formation (Arjonen et al. 2011). We subsequently investigated the effect of S3A mutation on prostate cancer cell adhesion to the extracellular matrix (ECM) component fibronectin and filopodia formation. The S3A mutation significantly increased cell adhesion to fibronectin compared to control WTCFL cells (Figure 4.8). This correlated with cytoskeletal remodeling as indicated by fluorescence staining of F actin and formation of filopodia (Figure 4.9). Confocal microscopy revealed an increased number of filopodia protrusions in S3ACFL PC-3 (arrows) compared to WTCFL PC-3 cells (Figure 4.9). High cofilin expression was detected at cell membrane regions populated by filopodia (Figure 4.10). Treatment with TGF- β led to a significant decrease in S3ACFL cell adhesion (Figure 4.9), and a reduction in filopodia protrusions (Figures 4.9 and 4.10).

To determine whether this cofilin co-localization with filopodia is dependent on endogenously derived TGF- β from the surrounding prostate cancer associated fibroblasts (reactive stroma), we subsequently profiled the cofilin/rhodamine phalloidin co-localization, in S3ACFL prostate epithelial cancer cells co-cultured with human CAFs. As shown on Figure 4.10, cofilin (green) colocalizes with filopodia protrusions (arrows) and loss of TGF- β resulted in increased actin/cofilin colocalization (yellow) with filopodia protrusions in S3ACFL cells in this reactive stroma-tumor microenvironment.

Figure 4.8 Active Cofilin Mediates Prostate Cancer Cell Adhesion via Cytoskeletal Remodeling, an Effect Impaired by TGF- β . The effect of S3A mutation on prostate cancer cell adhesion was assessed via cell adhesion assays to fibronectin. S3A mutation significantly increased cell adhesion to fibronectin compared to WTCFL control cells ($p=0.0003$). TGF- β treatment led to a significant decrease in S3CFL cell adhesion ($p=0.0004$), but no effect on WTCFL cells. Values shown are the mean (\pm SEM) of three independent experiments performed in triplicates. Statistical significance set at a P value of $p<0.005$.

Figure 4.8 Active Cofilin Mediates Prostate Cancer Cell Adhesion via Cytoskeletal Remodeling, an Effect Impaired by TGF- β .

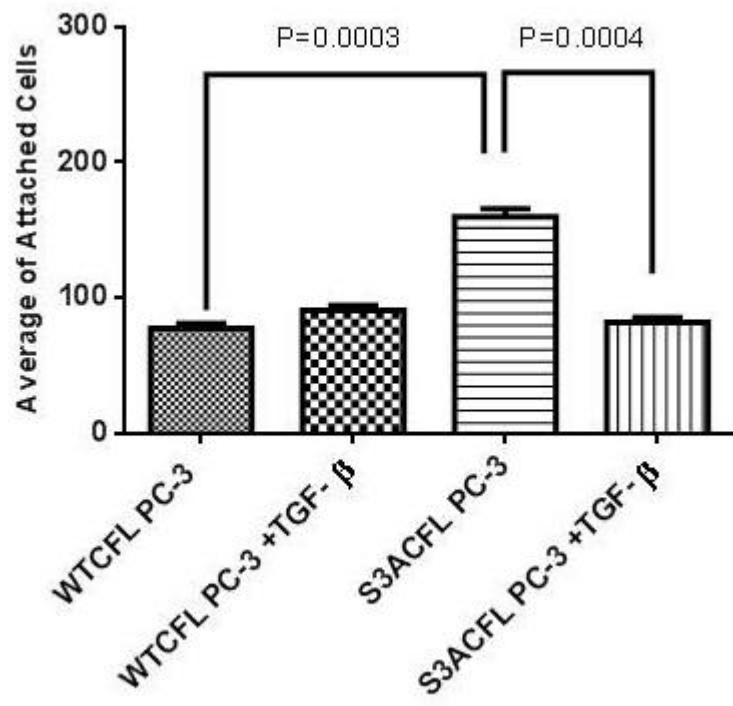


Figure 4.9 S3A Mutation Enhances PC-3 Filopodia Formation. The effect of S3A mutation on prostate cancer cells filopodia formation was assessed via Phalloidin staining of actin filaments. Active cofilin enhances filopodia formation; representative images of confocal microscopy (40x) show increased number of filopodia protrusions in S3ACFL PC-3 (arrows) compared to WTCFL PC-3 cells. Treatment with TGF- β (5ng/ml; 24hrs) decreased filopodia protrusions in S3ACFL cells. Filopodia were quantitated as we recently described (Zhu et al, 2012). Five random fields were examined for each cell line and values shown represent the mean \pm SEM from three independent experiments. Statistical significance is defined at $P < 0.01$.

Figure 4.9 S3A Mutation Enhances PC-3 Filopodia Formation.

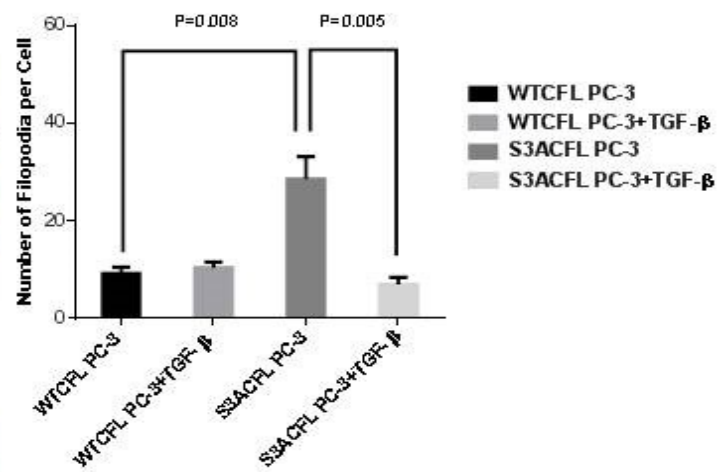
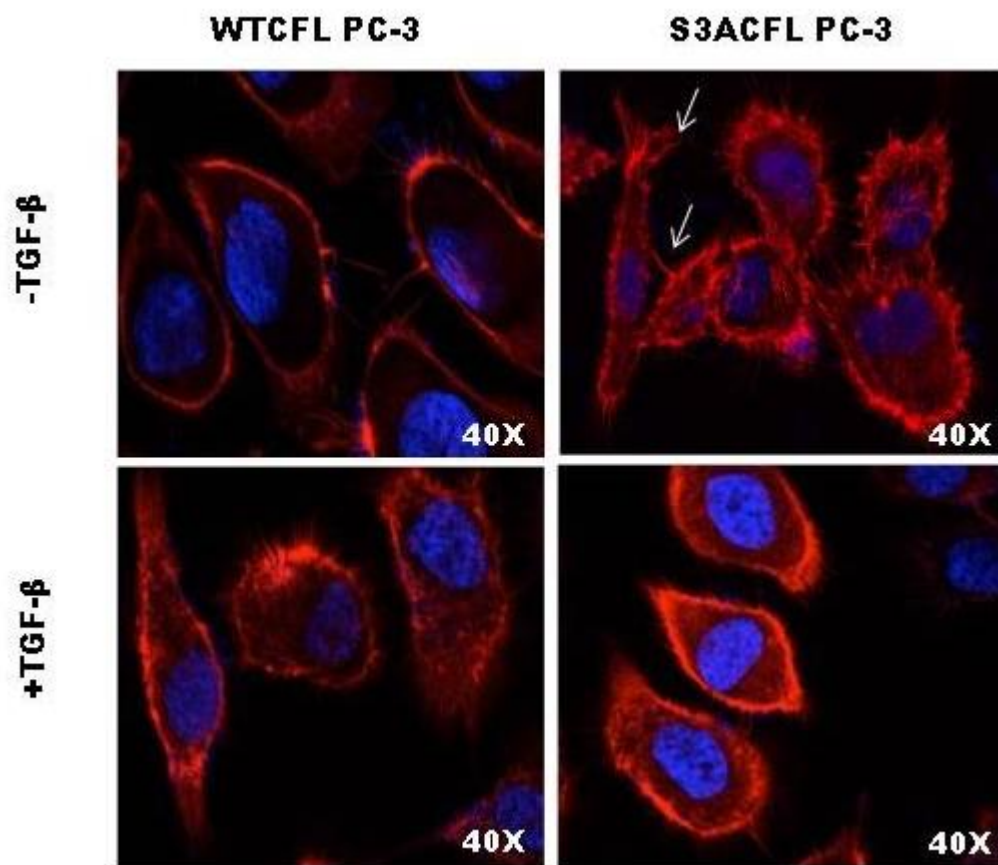
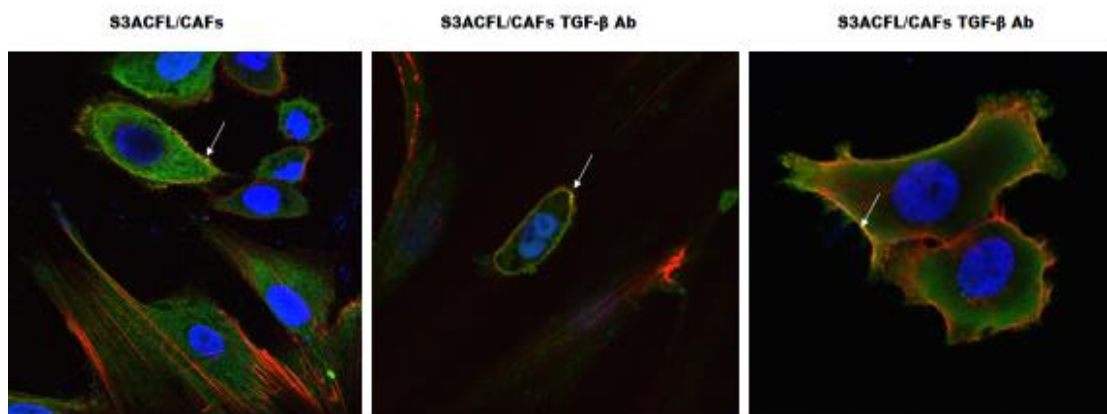


Figure 4.10 Cofilin Co-localization with Filopodia is Dependent on TGF- β Derived from The Surrounding Prostate CAFs (stroma). Images of cofilin/rhodamine phalloidin colocalization, in S3ACFL prostate epithelial cancer cells co-cultured with CAFs. Cofilin (green) colocalizes with filopodia protrusions (arrows). Loss of TGF- β (in presence of neutralizing antibody) resulted in increased actin/cofilin colocalization (yellow) and filopodia protrusions in S3ACFL cells.

Figure 4.10 Cofilin Co-localization with Filopodia is Dependent on TGF- β Derived from The Surrounding Prostate CAFs (stroma).



Active Cofilin Enhances Prostate Cancer Metastasis *In Vivo*

In the experimental metastasis assay, prostate cancer cells harboring the S3ACFL mutation exhibited an increased metastatic ability *in vivo*, compared to WTCFL cells, as determined by the higher number of lung metastases produced (Figure 4.11).

Figure 4.11 Cofilin Constitutive Activation Promotes Prostate Cancer Metastasis.

Panel A, Male nude mice (n=12) were inoculated with GFP-labeled PC-3 cells (parental, WTCFL and S3ACFL) via tail vein injections. Panel B, Metastatic lesions to the lungs were assessed at 4-wks post-inoculation. S3ACFL cells generated a significantly higher number of metastases compared to control PC-3 cells (p=0.04). Values show the number of metastatic lesions to the lung/mouse for each cell line. Western blots of mouse lung tissue homogenates and cell lysates indicate the GFP presence in all samples (positive control).

Figure 4.11 Cofilin Constitutive Activation Promotes Prostate Cancer Metastasis.

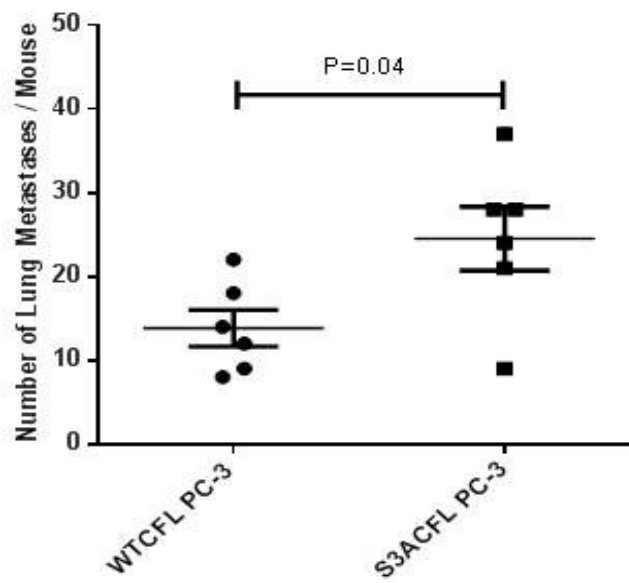
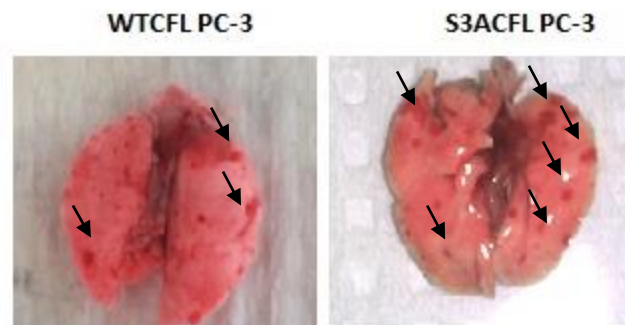
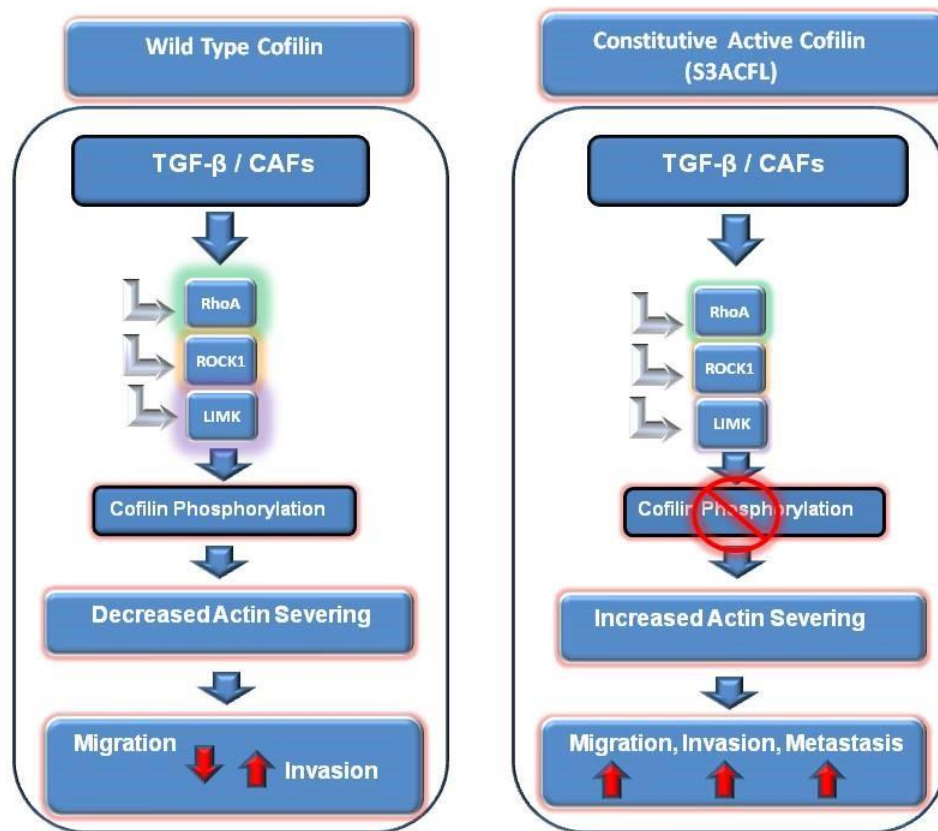


Figure 4.12 Schematic diagram illustrating the regulatory impact of cofilin on TGF- β functional switch towards prostate cancer cell migration, invasion and metastasis.

Under conditions of constitutive active (S3A mutant) cofilin, TGF- β produced by the reactive stroma/microenvironment (cancer associated fibroblasts), unable to dephosphorylate cofilin confers increased tumor cell aggressive characteristics and metastatic potential.

Figure 4.12 Schematic diagram illustrating the regulatory impact of cofilin on TGF- β functional switch towards prostate cancer cell migration, invasion and metastasis.



Cofilin Overexpression Correlates with Prostate Cancer Progression to Metastasis

TRAMP transgenic mice develop prostate adenocarcinoma with increasing age, resembling progression of human prostate cancer to metastasis. Analysis of cofilin expression during prostate cancer progression in the TRAMP model, revealed an association between high cofilin immunoreactivity and tumor aggressiveness with increasing age (16-28wks) (Figure 4.13). Quantitative analysis indicated a significant increase in cofilin expression in metastatic tumors (28-wks) compared to early stage tumors and normal prostate (16-wk WT) (Figure 4.13). Immunohistochemical profiling of cofilin in human prostate tissue specimens from a patient cohort with localized and metastatic disease to the lymph nodes, indicated a striking increase in cofilin expression in metastasis compared to primary cancer in the same patient (Figure 4.14). Characteristic images of cofilin immunoreactivity in poorly differentiated prostate tumors and metastasis are shown on Figures 4.14A and 4.14B, respectively). There were no significant differences in the expression of p-cofilin or palladin proteins between primary and metastatic prostate cancer (Figure 4.14, panels C and D).

Figure 4.13 Cofilin Profiling in TRAMP Mouse Model. TRAMP transgenic mice develop prostate adenocarcinoma with increasing age, resembling progression of human prostate cancer to metastasis. Prostate sections of increasing grade and metastatic tumors (16-28wks) were profiled by immunostaining for cofilin expression; WT mouse prostate tissue (16wks) was used as control. (magnification X40). Quantitative evaluation of cofilin immunoreactivity, as determined by the H-scoring, shows a significant increase in metastatic tumors from 28-wks old TRAMP mice ($p=0.001$) compared to early stage tumors.

Figure 4.13 Cofilin Profiling in TRAMP Mouse Model

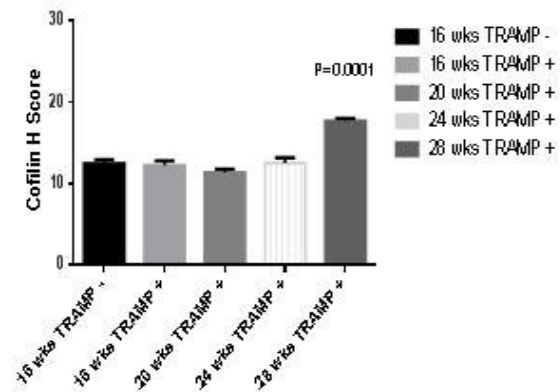
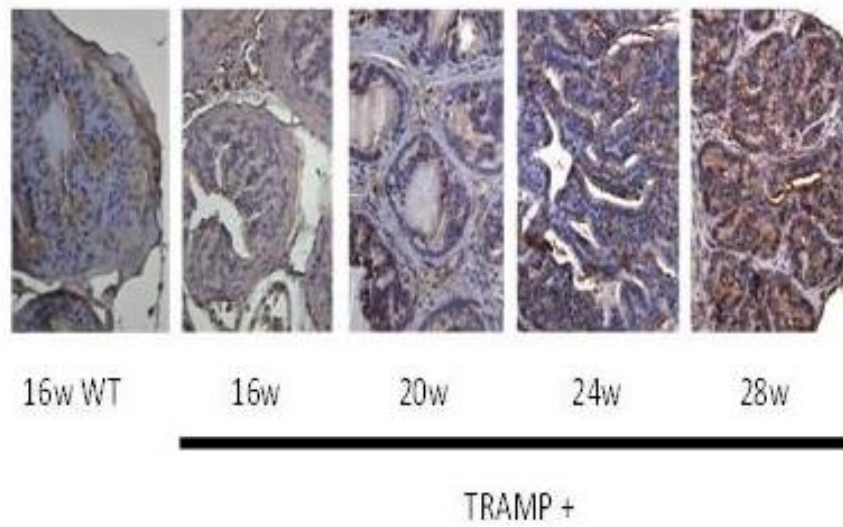
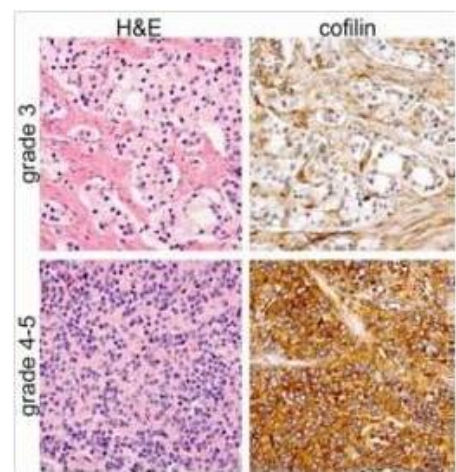


Figure 4.14 Cofilin Expression Profile in Human Prostate Cancer.

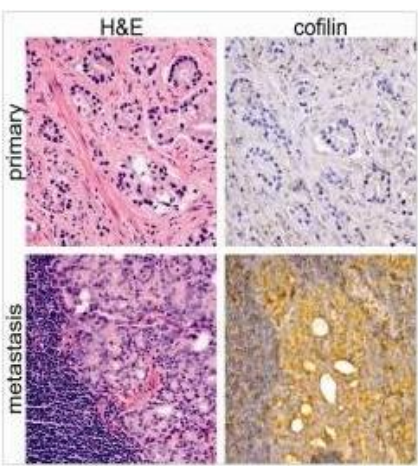
Panel A, H&E staining and cofilin immunostaining in serial sections of prostate tumors (Grade 3 and Grade 5) from two different patients. A striking increase in cofilin immunoreactivity was detected in the higher Grade prostate tumor. Panel B, characteristic image of a metastatic lesion to lymph nodes exhibiting intense cofilin immunoreactivity, compared to the primary tumor from the same patient (showing absence of cofilin expression). Magnification X, Panel C indicates representative images of immunostaining for cofilin, p-cofilin, E-cadherin and palladin on primary and metastatic prostate cancer. Panel D, quantitative analysis of protein immunoreactivity (from Panel C). There was significant increase in cofilin levels in prostate cancer metastasis compared to primary tumors ($p=0.005$).

Figure 4.14 Cofilin Expression Profile in Human Prostate Cancer.

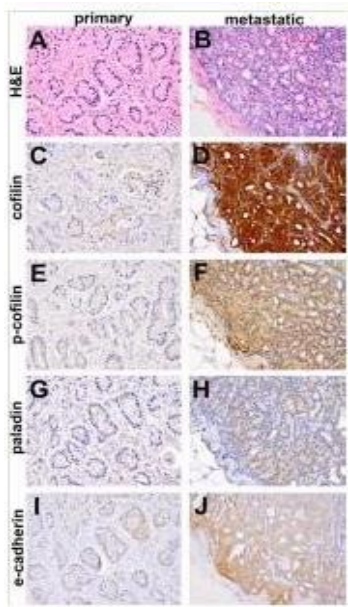
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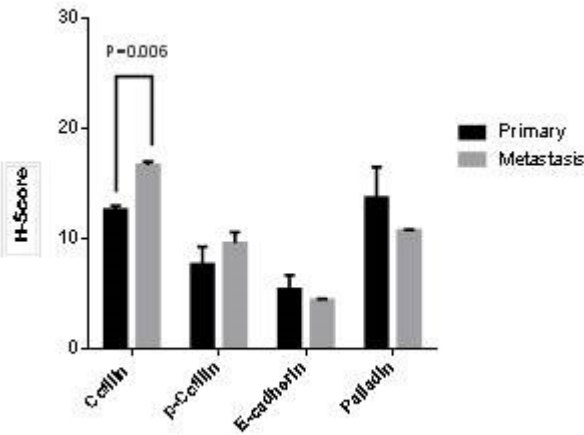
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D.



CHAPTER V

DISCUSSION

Cofilin has been previously identified as a Smad independent effector of TGF- β apoptosis signaling in prostate cancer cells (Zhu et al. 2006). TGF- β increases LIMK-2 activity (upregulates ROCK1 and RhoA kinases) leading to phosphorylated cofilin and decreasing actin cytoskeleton severing in prostate cancer cells. The present study indicates that while TGF- β mediates a striking reduction in the migratory capacity of WTCFL PC-3 cells, it fails to exert such an effect in the mutant S3ACFL cells (Fig. 4.12). As cofilin is unable to be phosphorylated by LIMK-2 (directed by TGF- β) in S3ACFL mutants, our findings suggest an alternative pathway via which TGF- β is modulating cofilin activity. One may argue that TGF- β signaling does not exclusively target modulation of cofilin severing activity, but it rather impairs prostate cancer in the early stages of disease progression by putting the “breaks” on cofilin activity (phosphorylation status). During the late stages of tumor progression, a mutation conferring constitutive activation of cofilin, enables escape from the TGF- β control of actin severing, towards enhanced migratory and invasive properties (Figure 4.12). This argument supports a sustained role of cofilin as an effector of TGF- β , potentially navigating its functional swinging from growth suppressor to a metastasis promoter during prostate tumorigenesis. An acquired enhanced motility at an early stage may provide S3ACFL mutants the initial input required to escape the primary tumor site, reach a nearby vascular tissue, intravasate, travel through the blood stream and

metastasize to a distal site. The optimal conditions will sustain colonization and growth and will allow prostate cancer cells to invade and proliferate into a secondary tumor. Via the secretion of cytokines and growth factors cancer cells now take advantage and modulate the new microenvironment to their favor supporting tumor growth and progression to metastasis. The high levels of TGF- β in the prostate microenvironment evidences the active tumor stroma dynamics. Cancer cells can secrete cytokines and respond to extracellular signals from the tumor microenvironment, however in response to TGF- β , cancer associated fibroblasts are going to differentiate into myofibroblasts which are known to be involved in extracellular matrix degradation facilitating the metastatic spread of adjacent prostate cancer cells. These dynamic interactions between cancer cells and the stroma microenvironment (inflammatory cells, vessels, fibroblasts and components of the ECM) in turn impact tumor invasion (Desmoulière et al 2004, De Wever and Marel 2003).

The results, described in this dissertation, support the notion that impairing cofilin activity (due to spontaneous mutations on phosphorylation site), is an early event promoting cancer cell migration and metastatic spread. The findings also indicate that cofilin severing activity towards actin cytoskeletal remodeling and increased prostate cancer cell-ECM adhesion and migration is dependent on TGF- β . Interestingly enough, we also report here that cancer associated fibroblasts substantially enhance the invasive properties of prostate cancer cells with mutant cofilin (constitutively active), regardless of TGF- β deprivation. This evidence provides a proof-of-principle on a direct pro-invasive crosstalk between surrounding cancer associated fibroblasts and prostate cancer cells with TGF- β functioning as a tumor suppressor by activating the RhoA/ROCK1 signaling,

leading to phosphorylation and activation of LIMK-2. This impairs cofilin severing activity, cytoskeletal reorganization and formation of filopodia, decreasing tumor cell migration (illustrated schematically on Figure 4.12). During prostate cancer progression the TGF- β functional switch from a growth suppressor to metastasis promoter, is programmed by activated cofilin that enables actin cytoskeleton remodeling, conferring aggressive tumor cell behavior (Figure 4.12).

Cancer metastasis is mediated by cell-matrix interactions engaging components of the extracellular matrix (ECM), to form adhesion complexes and actin polymerization to form cell protrusions to adhere to ECM, directing cell migration. Characterization of the actin cytoskeleton dynamics in tumor metastasis will enable a new platform for targeting significant protein interactions towards impairing metastatic progression, as well as identification of new markers of therapeutic response in advanced diseases. This study identified the functional contribution of cofilin to the metastatic process in prostate cancer. The results revealed significant differences in actin remodeling proteins, migration, invasion and adhesion potential between the wild type and mutant (constitutively active) S3ACFL PC-3 harboring a mutant cofilin phosphorylation site. S3ACFL conferred an increase in the migration potential compared to wild type PC-3 cells, suggesting that cofilin regulation is linked to acquisition of an enhanced migratory phenotype of prostate cancer cells. Considering that cofilin is directly responsible for the remodeling of actin filaments and filopodia formation toward cytoskeletal reorganization, ultimately driving cell motility, the finding that neither mutation S3A or T25A had any significant consequences on prostate cancer cell invasion might not be surprising. Indeed, cell migration relies on the coordinated remodeling of the actin cytoskeleton and leading

edge protrusions of moving cells are formed by lamellopodia and filopodia. (Arjoen et al. 2011). In breast cancer cells, cofilin activation by epidermal growth factor (EGF), leads to increased number of actin filament barbed ends. It is the elongation of barbed ends via the polymerization of G actin monomers that generates new actin filaments and dynamic filament branching at the tip of the leading edge (Zebda et al. 2000). In this study we found a marked increase in filopodia formation in S3ACFL PC-3 cells and lack of actin association with constitutively active cofilin, suggesting alterations in cofilin phosphorylation/dephosphorylation in prostate cancer cells interfere with its function in actin severing. Considering that filopodia can enable not only cell motility, but also facilitate attachment to the ECM and to a distal site promoting colonization and formation of secondary tumors (Arjonen et al. 2011), our findings support the concept that mutational activation of cofilin, besides enhancing cell movement, can also promote cell attachment to fibronectin, possibly by remodeling critical cell-ECM adhesion sites and regulatory protein associations.

This study establishes that constitutively active cofilin results in actin cytoskeleton remodeling impacting prostate cancer cell adhesion, migration and invasion in response to TGF- β . Cofilin is thus a non-canonical effector of TGF- β signaling, capable of coordinating the cellular responses to TGF- β towards metastasis. The significant association between cofilin overexpression/activation with prostate tumor invasive and metastatic behavior, supports a potential predictive and targeting value for cofilin in cancer metastasis.

The immunohistochemical profiling identified a direct association between cofilin overexpression and cancer progression to metastasis in the TRAMP mouse model of

prostate tumorigenesis. Moreover, my studies demonstrate a significant increase in cofilin expression in human prostate cancer metastasis (to lymph nodes), compared to primary tumors. Changes in cofilin expression have been reported in other human malignancies including colon and ovarian cancer (Wang et al. 2007, Sadako et al. 2010, Popow et. al 2012). Loss of the epithelial marker E-cadherin is associated with a more invasive phenotype in prostate cancer cells and high grade and metastatic prostate cancer, as previously established (Umbas et al. 1992). In addition immunohistochemical profiling of total cofilin in tissue microarrays (TMAs) from breast and colon cancer showed high levels of expression of total cofilin supporting again, a potentially significant value for cofilin as a biomarker not only for prostate cancer but for different types of cancers. Ongoing studies in collaboration with Dr. Andre Balla at the University of Illinois, include the profiling of total cofilin and phosphorylated cofilin in TMAs from a larger prostate cancer patient cohort with advanced disease.

We should consider the involvement of additional actin binding proteins acting together to facilitate actin cytoskeletal remodeling since cofilin severing activity is not sufficient to support the *de novo* synthesis of actin filaments at the leading edge of moving cells. Without actin nucleation by the Arp2/3 complex, the presence of actin bundling proteins and the addition of actin monomers by profilin, cytoskeletal remodeling will not progress. We found an increase in the actin bundling protein plectin together with cofilin in metastatic human prostate cancer specimens compared to primary prostate cancer from the same patient, supporting the role of additional actin remodeling proteins in the progression of prostate cancer towards metastasis. Since CAFs, or activated fibroblasts present in the stroma surrounding solid tumors are capable to promote

invasion and metastasis of cancer cells, the mechanisms regulating the activation of the fibroblasts and the initiation of invasion are of great interest. Interestingly, the upregulation of the cytoskeletal protein, palladin, has been found to be upregulated in stromal myofibroblasts surrounding many solid cancers and in expression screens for genes involved in invasion. Studies involving a pancreatic cancer model, investigated the functional consequence of overexpression of exogenous palladin in normal fibroblasts *in vitro* and its effect on the early stages of tumor invasion. These studies demonstrated that that palladin expression can impart myofibroblast properties, in turn promoting the invasive potential of these cells with invadopodia-driven degradation of extracellular matrix (Brentnall et al., 2012). Additional elegant studies, have confirmed that the conversion of fibroblasts into active cancer associated fibroblasts also called myofibroblasts, not only is induced by TGF- β , but also involve an increase in the expression of palladin (Rönty et al. 2006). The secretion of extracellular proteins, proteases, cytokines, and growth factors by myofibroblasts results in the modulation of the ECM (Powell et al., 1999; Tomasek et al., 2002). We can consider a scenario in which TGF- β in the tumor microenvironment mediates the upregulation of palladin not only in tumor associated fibroblasts favoring their conversion into myofibroblasts and the degradation of the ECM but also the upregulation of palladin in cancer cells. Upregulation of the actin bundling protein palladin, together with an upregulation of cofilin severing activity, may provide prostate cancer cells with enhanced actin remodeling activity facilitating their escape into the stroma, once the ECM barrier have been disrupted by active myofibroblasts. While TGF- β 's role in myofibroblastic differentiation have been widely studied, the signaling pathways involved in cytoskeletal

modulation are not well characterized. My work identified for the first time that TGF- β induced expression of palladin and cofilin is regulated via signaling pathways targeting cytoskeleton remodeling in prostate cancer cells. These results are of high clinical significance since the use of biological markers for better prognosis and treatment of prostate cancer patients relies on the identification of specific proteins correlating with metastatic potential. The immunohistochemical analysis for the expression of cofilin in primary human prostate cancer tissue together with the actin bundling protein palladin and the presence of active myofibroblasts via α -SMA staining have the potential to be exploited as a novel and patient specific tool for predicting prognosis in prostate cancer patients. The overexpression of unphosphorylated (active) cofilin, together with the overexpression of palladin and the presence of activated fibroblast at the primary tumor can be used as a marker for prostate cancer metastatic burden. Therefore, I am proposing the development of a novel screening technique involving not only the screening for prostate specific antigen PSA at early stages of prostate cancer but in combination with cofilin, palladin and α -SMA biomarkers. This technique will supplement PSA screening and allow to better predict which patients are at higher risks of developing metastatic prostate cancer and will require a more aggressive treatment. Indirect support for this approach is gained from recent evidence correlating cofilin expression with ovarian cancer progression, and a longer progression free survival in low cofilin patient cohort (Nishimura et al. 2011) together with findings showing that paladin is overexpressed in the CAFs of several tumor types including pancreas, breast, lung, kidney, and ovary but is expressed at lower levels in normal stromal fibroblasts (Goicoechea et al., 2010, Ronty et al., 2006).

In addition to the potential use of cofilin as a potent biomarker for predicting prognosis, the use of cancer specific RhoA and ROCK1 kinase inhibitors for the temporally regulation of cofilin severing activity will allow to inhibit prostate cancer metastatic spread at early stages of the disease without compromising the overall cofilin activity which is necessary for the survival and growth of non-cancer cells. Via the inhibition of RhoA and ROCK1 kinases at early stages of the disease in mutant cancer cells, cofilin's phosphorylation by LIMK in response to TGF- β is blocked, suppressing cofilin enhanced severing activity and the development of an enhanced migratory phenotype thus suppressing metastatic spread. Our results revealed a suppression of the RhoA/ROCK1 signaling pathway in the wild type cells, however mutant S3ACFL being unable to be phosphorylated by LIMK in response to TGF- β were able to escape its tumor suppression. Another therapeutic approach based on our results would be the use of genetic screening to determine patients with mutations in cofilin phosphorylation site predisposing these patients to an aggressive cancer due to the loss TGF- β tumor suppression at early stages of the disease. This approach would allow a better understanding of prostate cancer dynamics at early stage of disease for each individual patient and will translate in the development of personalized treatment depending on the genetic background, in terms of cofilin mutations that could facilitate cell motility and adhesion to the ECM, together with mutations regulating intercellular adhesion, such as like E-Cadherin and N-Cadherin, as well as cytoskeletal ECM linking proteins such as integrins.

An insight into each of the steps preceding metastatic spread reveals a common denominator for the majority of primary solid tumors; the loss of cell to cell adhesions,

detachment from the basement membrane and the acquisition of an enhanced migratory phenotype; each of these events being directly dependent on cytoskeletal changes and ECM remodeling. It is not surprising for the cell cytoskeleton to play such important role in the determining the fate of cancer since it comprises the most extended network for communication between signaling proteins inside and outside a cell. It is the constant traffic of signals facilitated by motor proteins such as Dynein through the cell cytoskeleton what facilitates cell movement in any tissue environment. Although many efforts focused on the tumor suppressor and oncogenic pathways in cancer allowing specific cell types to undergo malignant transformation, we must always recognize that most of prostate cancer patients do not die from primary tumors and less attention have been directed to the cytoskeletal changes allowing cancer cells to undergo metastasis spread. Our focus on the main regulator of the cytoskeleton dynamics cofilin allow us to explore many alternatives to not only block the overall migratory potential of prostate cancer cells but the transport of signaling proteins and the transduction of signaling cascades that facilitates the process of cancer metastasis. One of the possibilities includes as discussed before, targeting cofilin activity for the suppression of cancer migration at an early stage, however another possibility includes the regulation of cofilin severing activity spatially for the delivery of specific therapeutically agents along the cytoskeletal network. By modulating the activity actin binding proteins like cofilin and palladin we can attain the selective remodeling of the cellular actin network to our advantage, the same way cancer cells remodel the cytoskeleton to obtain an enhanced metastatic potential. Therapeutically this will translate into the possibility to selective transport drugs such as small molecular inhibitors to specific targets inside the cell or to block

signaling molecules like transcription factors from reaching their targeted genes many of them tumor promoter genes and oncogenes promoting tumor progression.

One of the most important candidates for this therapeutic technique based on cytoskeletal modulation and transport would be the AR. Via the disruption of nuclear actin network in the same way microtubule-targeting drugs, such as the *Vinca* alkaloids and taxanes, have been used to target the mitotic spindle checkpoint, arresting cell cycle progression leading to apoptosis it could be possible to attack castration resistant tumors via the disruption of AR translocation into the nucleus. To support this idea, previous studies from our group comparatively analyzed TMAs from Docetaxel-treated and untreated prostate cancer patients for prostate specific antigen (PSA) and AR immunoreactivity. The study revealed that in addition to blocking cell division, the microtubule stabilizing drug Docetaxel impairs AR translocation into the nucleus (Zhu et al., 2010). Although it is clear that microtubules and actin cytoskeleton have distinct roles, there have been studies that evidence an interaction between these two, moreover it have been found that an intact microtubule cytoskeleton is needed to maintain the polarized distribution of actin protrusions at the leading edge of migrating fibroblasts (Vasiliev et al., 1970). Thus the targeting microtubules/actin interactions emerges as a novel therapy not only for the suppression of cancer cell movement and progression to metastasis but for the delivery of drugs into the cell without affecting overall toxicity and overcoming drug resistance via the modulation of signaling cascades routes. The best evidence supporting the interactions between the microtubule and actin network is provided by the Rho family of GTPases, which can regulate both actin filaments and microtubules (Wittmann and Waterman, 2001). Based on our results we can propose a

mechanism in which RhoA can suppress the actin polymerization while promoting the stabilization of microtubules at the same time via the activation of ROCK1 kinase resulting in the phosphorylation of cofilin and the inhibition of actin filaments which in turns help stabilize the microtubule network. Thus, we can submit the notion that the activity of RhoA is regulated in the same time by microtubules and actin. The activation of cofilin severing activity and actin polymerization will promote RhoA/ROCK1 leading to phosphorylation of cofilin and the inhibition of actin polymerization resulting on microtubule stabilization. Microtubule stabilization will inhibit RhoA /ROCK1 in a negative feedback reactivating cofilin severing activity. By transiently inhibiting RhoA/ROCK1 we can stimulate cofilin activity, stabilizing microtubule and altering the microtubules tracks required by protein effectors for the transduction of signaling cascades (Figure 5.1).

Figure 5.1 Potential Mechanism for Microtubule Stabilization and Transcriptional Inhibition via Targeting of Cytoskeletal Remodeling. RhoA activation suppresses actin polymerization while promoting microtubules stabilization at the same time via the activation of ROCK1 kinase resulting in the phosphorylation of cofilin and the inhibition of actin filaments which in turns help stabilize the microtubule network. Microtubule stabilization results in the inhibition of signaling molecules and transcription factors from reaching their targeted genes leading to tumor suppression.

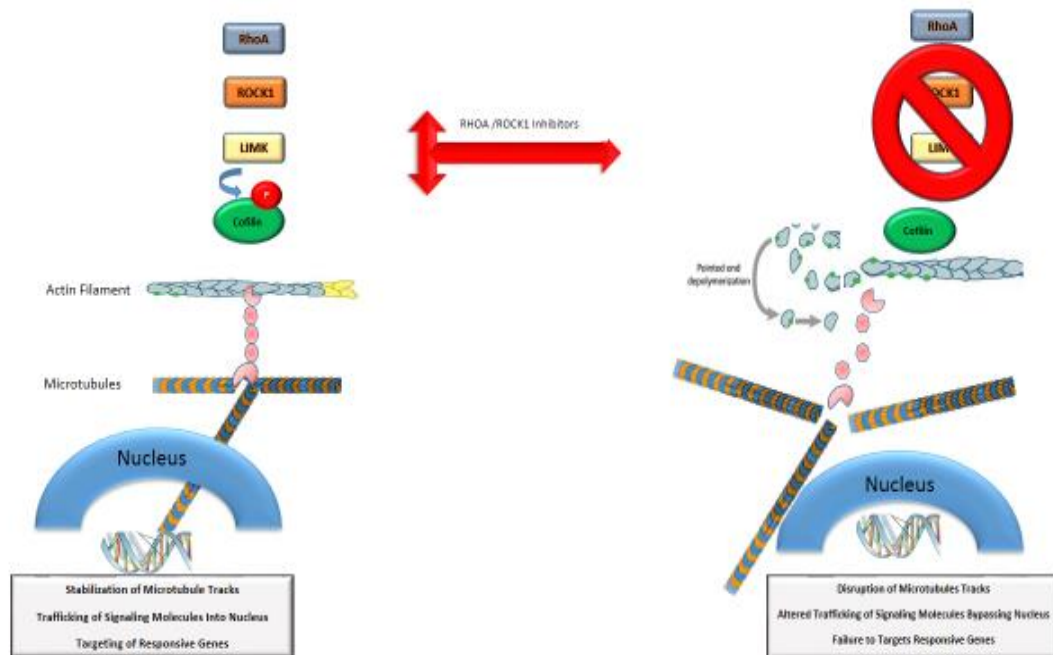


Figure 5.1 Potential Mechanism for Microtubule Stabilization and Transcriptional Inhibition via Targeting of Cytoskeletal Remodeling

Cell to cell communication also play critical role on cancer progression. A collection of cell surface proteins recognize signals from the microenvironment and nearby cells. Filopodia structures not only are rich in cell adhesion proteins, they are responsible for probing the pericellular environment for chemotactic factors and other molecular signals in the ECM that enable and direct the movement of the cell and for receiving and transmitting information between cells the same way dendrites are used by neuronal cells to receive and conduct the electrochemical stimuli from other neural cells inside the brain (Horace, 2011). This work demonstrates that the modulation of cofilin actin severing activity affects the development filopodia protrusions of prostate cancer cells, a significant discovery, since cells utilize filopodia to communicate within the tumor microenvironment towards metastasis. Our results demonstrated that a mutation on cofilin phosphorylation site (S3A) can enhances filopodia protrusions bypassing TGF- β tumor suppression. This study reveals for the first time that cytoskeletal changes impact the ability of prostate cancer cells to recognize signals from the tumor microenvironment. Proteomics studies performed in collaboration with Dr. Haining Zhu, demonstrated an overexpression of EMMPRIN (extracellular matrix metalloproteinase inducer) in the cell surface of prostate cancer PC-3 cells compared to benign BPH-1 (Zhu. et al, 2011). EMMPRIN silencing markedly impaired cancer cell adhesion and filopodia formaton. One may consider that the overexpression of EMMPRIN result as cancer cells develop a more aggressive and motile phenotype and develop filopodia. Each filopodium serves as a template for the cell surface protein EMMPRIN to be exposed to be recognized by nearby cells recruiting cells bearing the EMMPRIN receptor on the cell surface in the the

same way as immune cells are recognized by and communicate with antibodies in the circulation by exposing their epitopes at the cell surface (Figure 5.2).

Figure 5.2 Role of Filopodia in Tumor Microenvironment Signal Recognition and Transduction. The development of filopodia structures in cancer cells provides a template for the cell surface protein EMMPRIN to be exposed and be recognized by nearby cells, recruiting cells expressing the EMMPRIN receptor to the tumor site. Recruitment of ECM remodeling cells like CAFs, leads to ECM degradation and tumor invasion.

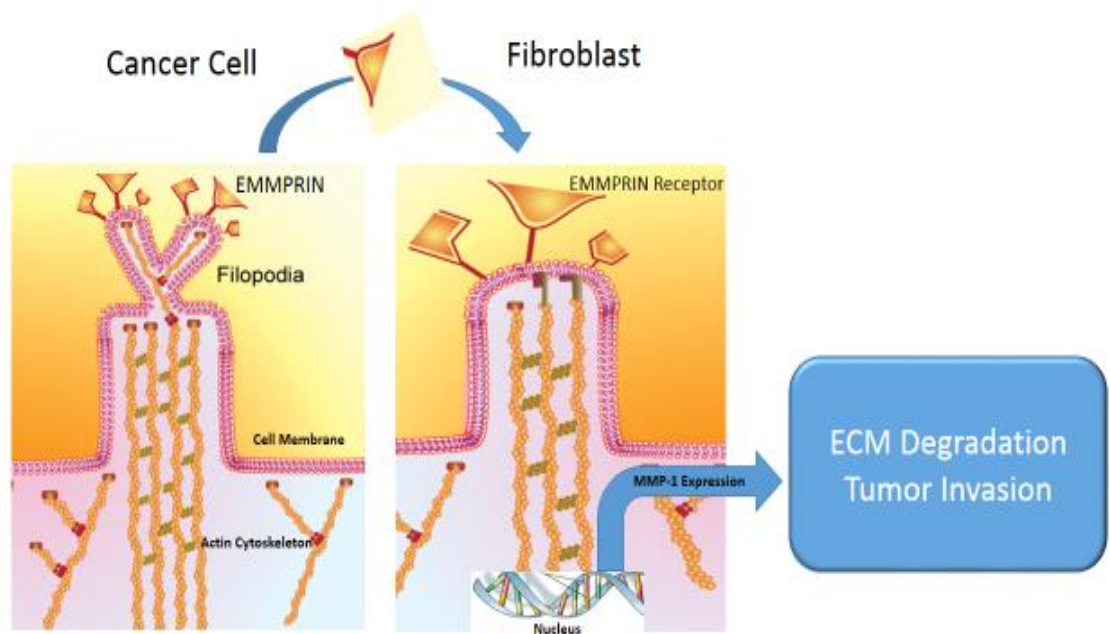


Figure 5.2 Role of Filopodia in Tumor Microenvironment Signal Recognition and Transduction.

What is more, the EMMPRIN interaction with its receptor on target fibroblasts, is known to upregulate MMP-1 (matrix metalloproteinase 1) transcription facilitating tumor invasion and metastasis (Sidhu et al., 2004). Consistent with these findings we have shown enhanced invasion of prostate cancer cells in the presence of cancer associated fibroblasts. By targeting cofilin severing activity and cellular cytoskeleton remodeling we can impair the development of filopodia on tumor cells inhibiting EMMPRIN exposure, the recognition by cancer associated fibroblasts and their recreation to the tumor site, therefore inhibiting ECM degradation, tumor invasion and metastasis. What is more, studies have demonstrated that therapeutic treatment of pancreatic cancer that reduces the cancer-associated fibroblasts is more effective in prolonging survival than standard chemotherapy that targets only the cancer cells (Olive et al., 2009, Sahai, 2010, Xu et al., 2010). Thus deviating signaling cascades to inhibit the recruitment of fibroblast to the site of cancer via the modulation of cytoskeletal remodeling, can be exploited as a pre - chemotherapeutic treatment to prolong patient survival. The above therapeutic approach will change the way we look at and treat cancer today. Since 1889, the seed and soil theory has proposed that the presence of factors in specific organ are responsible for the growth of only certain types of cancers. Based on my findings, I proposed a new hypothesis in which there is not the presence of unique factors in the site of metastasis, that promote selective metastatic spread to a specific organ but, the ability of cancer cells to receive, transduce and alter signaling pathways on nonmalignant cells in the new tumor site. Once cancer cells has taken control of the new microenvironment they can signal non-malignant cells to secrete growth factors and immunosuppressive cytokines

needed by cancer cells to grow and proliferate on a foreign environment. This study proposes that the future of cancer therapy, could rely on the targeting of molecular pathways leading to the development of specific signaling structures such as the cofilin signaling pathway to block filopodia structures and the target of cell surface signaling proteins like EMMPIRIN. Thus by blocking the ability of cancer cells to recognize, transduce and deliver signals to the tumor site, we should be able to indirectly block, not only the process of EMT, ECM degradation and invasion, intravasation into circulation at early stages but the recruitment of blood vessels via stimulation of VEGF, and responses to growth factors stimuli like TGF- β on already established tumors, changing the fate of tumor cells to die rather than grow and proliferate.

Clinical Significance

For many years, prostate specific antigen (PSA) screening have saved many lives, since it translated on early prostate cancer detection and treatment. However many times it has also led to over-diagnosis and overtreatment of prostate cancer patients (Etzioni et al., 2012). Another critical problem about the use of PSA is the limited predictive accuracy for predicting outcomes after treatment and for making clinical decisions about the type and intensity of therapies. Although Gleason Score information have been used by pathologists to understand how a particular case of prostate cancer can be treated and patients likely to survive following a diagnosis of prostate cancer, this approach is based exclusively on the architectural pattern of the glands of the prostate tumor, does not provide information on therapy selection and does not count with a mechanistic

foundation that can guide the best sequences or combinations of agents in targeting specific biomolecules. As a result, patients are currently grouped by clinical stage or treatment status as: with or without bone metastasis, resistance to androgen ablation therapy or not, with or without chemotherapy (Logothetis et al. 2013). Even though the patient's Gleason score with his PSA level and the clinical stage estimated by the physician can be used to estimate the likelihood that that patient has localized or locally advanced prostate cancer of different types, there is an imperative necessity for new molecular markers that define a specific stage of progression for the selection of the appropriate therapeutic approach independently of tumor stage. This work represents a whole new era of prostate cancer screening and management, taking in consideration a panel of biomarkers including, genetic background, protein expression at the molecular level to predict prognosis and therapy selection as well as the targeting of the communication network between biological components and the active tumor microenvironment. All of this valuable information will supplement the use of PSA and Gleason Scoring and will lead to a better understanding of prostate cancer that will be used to attain sufficient degree of certainty that would guide the best clinical decisions based on individual patients.

Future Directions

In order to understand the role of cofilin in normal prostate epithelial cells, a mutant S3ACFL, benign prostate hyperplasia (BPH) cells line will be generated via stable transfections and migration, invasion and cell adhesion ability will be compared between wild type and mutant cofilin BPH cell lines.

To examine if TGF- β signaling is required for the suppression of prostate cancer metastasis on mutant S3ACFL and WTCFL PC-3 cells at the physiological level, the double transgenic DNT β RII/TRAMP mouse model bearing a dysfunctional TGF- β type 2 receptor will be injected with wild type and mutant cofilin PC-3 cells via tail vein and metastatic lesions to the lung will be analyzed. This study will allow to characterize the *in vivo* consequences of an inactivated TGF- β signaling on mutant S3ACFL and WTCFL PC-3 cells progression to metastasis. The results will show if a dysfunctional TGF- β signaling mechanism results in loss of the inhibitory effects of TGF- β leading to an increase of prostate cancer epithelial cell metastatic potential.

As a secondary model of tumor metastasis, we will use an orthotopic implantation model of human prostate cancer with the purpose of mimicking closer some of the characteristics of human cancer metastasis. This model consist on the direct implantation of PC-3 cells into the ventral lobe of the prostate of athymic mice allowing tumors to progress for 4 to 6 weeks. At experiment termination, several distinct endpoints will be measured, such as size and molecular characterization of the primary tumor in terms of total and phosphorylated cofilin expression, the presence and quantification of circulating tumor cells in the blood and bone marrow, and number of metastatic lesions to the lung.

To further investigate the role of cell surface plasma membrane proteins in the process of prostate cancer metastasis, we are also interested in the development of a double transgenic TRAMP/EMMPRIN knockout mice, to elucidate the effect of loss of the cell surface protein EMMPRIN in prostate cancer progression to metastasis.

APPENDIX

LIST OF ABBREVIATIONS

AR- androgen receptor
ATP- adenosine triphosphate
BPH- benign prostate hyperplasia
CDKs- cyclin dependent kinases
CFL- Cofilin
CRPC- Castration resistant prostate cancer
DAB- Diaminobenzidine
CAFs- Cancer Associated Fibroblasts
CFL- Cofilin
DNA- Deoxyribonucleic Acid
DNTGFRII- Dominant negative Transforming Growth Factor Beta Type II Receptor
EMMPRIN- Extracellular Matrix Metalloproteinase Inducer
ECM- Extracellular Matrix
EDTA- Ethylenediaminetetraacetic acid
EGFR- Epidermal growthfactor receptor
EMT - Epithelial to Mesenchimal Transition
ECM - Extracellular Matrix
EDTA - Ethylenediaminetetraacetic acid
FGF- Fibroblast growth factor
GFP- Green Fluorescent Protein
LIMK- Lim domain kinase
MAPK- Mitogen- activated protein kinase
MTT- (3-(4,4,5-Dimethylthiazol-2-yl)-2,5)-diphenylterazolium bromide

PCR- Polymerase Chain Reaction

PBS- Phosphate Buffered Saline

PI3K- Phosphatidylinositide 3-kinases

PMSF- Phenylmethanesulfonylfluoride

PSA- Prosta Specific Antigen

PTEN - Phosphatase and tensin homolog

RANKL- Receptor activator of nuclear factor kappa-B ligand

RIPA- Radioimmunoprecipitation Assay Buffer

ROCK1- Rho-associated, coiled-coil containing protein kinase 1

SDS- PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SHH- Slingshot phosphatase

TGF- β - Transforming growth factor beta

TMPRSS2- Transmembrane protease, Serine 2

TRAMP- Transgenic adenocarcinoma of the mouse prostate

UGM- Urogenital sinus mesenchyme

UGS- Urogenital sinus

VEGF- vascular endothelial growth factor

VEGF- vascular endothelial growth factor receptor

WT- wild type

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EDUCATION

2008 - present Ph.D. Toxicology, University of Kentucky School of Medicine, Lexington, KY

1997 - 2003 BS General Sciences, University of Puerto Rico, Rio Piedras, Puerto Rico.

PROFESIONAL POSITIONS

Present Position

Graduate Research Assistant. University of Kentucky, Department of Toxicology, Dr. Natasha Kyprianou Mentor, University of Kentucky Markey Cancer Center (NCI).

Previous Positions

Research Assistant. University of Puerto Rico, Rio Piedras, Puerto Rico
2000

Research Assistant. University of Puerto Rico, Rio Piedras, Puerto Rico
2001

Regulatory Affairs Assistant. Procter & Gamble Laboratories, San Juan, Puerto Rico
2004

Assistant Biologist. Department of Natural Resources San Juan Puerto Rico and Federal Fish and Wildlife Services US

2005

Graduate Research Assistant. University of Kentucky, Kentucky, US

2007

Awards and Honors

Academic Excellent Award for Undergraduate Studies

1997

Lyman T. Johnson Academic Year Fellowship Award

2008

Lyman T. Johnson Academic Year Fellowship Award

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Lyman T. Johnson Academic Year Fellowship Award

2010 AUA Summer Research Conference Travel Award, Baltimore, US

2012

2012 SBUR Fall Symposium Travel Award, Miami, US

2012

NATIONAL AND INTERNATIONAL TEACHING ACTIVITIES AND INVITED LECTURESHIPS

Mentored student (8-week research project): The Puerto Rico Toxicology Research Summer Program.

Graduate Center for Toxicology, University of Kentucky, School of Medicine, University of Kentucky, Kentucky, US

2011

Presented Lecture: (lecture presented to medical school residents). Significance of Cytoskeleton Reorganization in Prostate Cancer Progression to Metastasis. Department of Urology, University of Kentucky School of Medicine, Kentucky US.

2012

Podium Presentation: Significance of Cytoskeleton Reorganization in Prostate Cancer Progression to Metastasis. Graduate Center for Toxicology, University of Kentucky, and School of Medicine, Kentucky US.

Podium Presentation: Cofilin, A Cytoskeleton Regulator Mediating TGF- β effect in Prostate Cancer Metastasis. Society for Basic Urologic Research, 2012 Fall Symposium, Miami Florida US.

2013

Presented Lecture: (lecture presented to senior high school students). Pursuing a Career in Cancer Research. Petra Corretger De O'Neill High School, Manati, Puerto Rico.

2013

Podium Presentation: Cofilin, A novel regulator of Prostate Cancer Metastasis. First International Conference in Anticancer Research, Fourth International Conference on Recent Advances in Health and Medical Sciences, Twenty second International Conference on Chelation, Azzia Resort and Spa Paphos, Cyprus .

2013

PEER-REVIEWED PUBLICATIONS

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